

INHIBITING THE MASTER REGULATOR, HEAT SHOCK TRANSCRIPTION  
FACTOR (HSF1) USING A POTENT RNA APTAMER IN *DROSOPHILA*  
MELANOGASTER & HUMAN CANCER CELLS

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by

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Cornell University 2009

**Abstract:**

All organisms have a well-conserved heat shock response that is universal among species as diverse as bacteria, plants, and animals. Upon heat stress, the heat shock transcription factor (HSF1) orchestrates the expression of molecular chaperones that allow the organism to cope with the cellular damage induced by the stress. Interestingly, these molecular chaperones, or heat shock proteins, are amongst the most highly conserved proteins throughout bacteria, animal and plant kingdoms. Because a high concentrations of damaged proteins is deleterious to cells, the heat shock response has become an integral survival response of living systems, one that rapidly evolving systems like cancer cells utilize quite efficiently. Therefore, to further understand genetic instability disorders like cancer, and at the same time to broaden the identification of novel drug targets, we must first be capable of efficiently disrupting the chaperone-buffering system that maintains cancer cell survival.

Recently, HSF1 has been found to promote cellular transformation in mammals. My studies focus on the role of HSF1 in animal development and maintenance of the transformed phenotype using a highly-specific RNA

aptamer (iaRNA<sup>HSF</sup>) that binds to yeast, *Drosophila*, and mammalian HSF1. In *Drosophila*, the expression of iaRNA<sup>HSF</sup> reduces normal expression of the *Hsp83* chaperone and induces developmental abnormalities that mimic the spectrum of phenotypes previously reported when Hsp83 activity is reduced. Using *Drosophila* mutants as a model for tumorigenesis, I found that HSF1 inhibition with iaRNA<sup>HSF</sup> effectively suppresses the abnormal growth phenotypes induced by constitutively active EGF receptor (EGFR) and Raf oncogene that normally function as part of the Hsp83-regulated cell survival pathway. Moreover, HSF1 inhibition by this same iaRNA<sup>HSF</sup> in various human cancer lines resulted in the reduction of molecular chaperones that normally promote cell survival, thereby triggering apoptosis. Collectively my studies demonstrate the potent application(s) of the RNA aptamer technology in investigating transcription regulation and cancer biology, as well as in testing of putative drug targets *in vivo*.



## BIOGRAPHICAL SKETCH

As a child, I grew up not knowing my real parents. I remember my Grandmother, Maria Teresa, constantly reminding my older sister Krissia and me that someday my parents were going to come back and take us to the land of opportunities, “Los Estados Unidos”. Not knowing my parents did not really bother me. On the contrary, I was glad that they were far away, because I knew that if they had stayed they would be buried in some dump somewhere in the highlands.

During the late 1970's, both of my parents were students in La Universidad Nacional de El Salvador, when the government shut down the universities and began to assassinate those professors and students that spoke against the injustices, which had been imposed by the fourteen families that controlled every aspect of the Salvadorean government and economy. Due to this event, my father was forced to quit on his third year of medical training. One day, a soldier came up to my father and asked him if he could take a picture of him with his “beautiful” family: my mom, who had me inside her womb, and my older sister, who was only just over one year old. Before my father could agree to this event, the soldier had already taken two pictures, one which was given to him, and the other picture which was property of the Salvadorean government, and ended up being posted in a bulletin board of suspected “outspoken” individuals, a mere assassination list. After my mother had given birth to me, both of my parents decided to leave the country, simply because many of their friends had been taken into custody for “questioning” and never returned to their families. In time, their body parts were found scattered throughout public places, including local Plazas. Afraid for their lives, my parents began the three thousand mile journey toward North America

in hope a better life, leaving my sister and me under my grandmother's care. In the years that followed, both of my parents worked in the strawberry, and broccoli fields of Southern California picking vegetables in order to send one hundred and fifty dollars a month to help my Grandmother for our care.

Growing up in Chinameca, in the outskirts of El Salvador in the height of the war meant that school was almost always cancelled. This did not stop my Grandmother, a retired school grade teacher, from attempting to give my sister and me a proper education. During her career as a school teacher, she had practically taught everyone in our "pueblo" how to read and write, and for this reason, everyone treated her with great respect and admiration. Without realizing it, I had joined the first grade already knowing how to read and write. During the nights, when the helicopters would fly over the hills dropping flares and bombs, my sister and I would read from old encyclopedias that my Grandmother had rescued from her school before it was burned to the ground during a battle. At nights, when the "guerrillas/rebels" would try to ambush the government soldiers, we would go into our man made shelter and fall asleep to the sounds of troops running at a quick pace right in front of our house, helicopters flying low, nearby cries, bombs exploding followed by the rapid gunfire of a screaming M16 rifle, pppp...ppppp....pppppp....pppp....pppppp. Some nights the fighting would cease during the night, while other times, it would continue for days.

One day, in the middle of the summer, my Grandmother came from the local telephone center excited. As soon as she entered the house, she looked in my direction and said, "your parents are on their way, they'll be here tomorrow!" I did not know what to think, I felt happy that I was going to finally see my parents, at the same time I could not disguise my sadness, because I

knew that the day had finally arrived where I was going to leave everything I knew, everyone I loved, my family, my dog, my home. My Grandmother, on the other hand seemed very happy, for it had been ten years since she had last seen her daughter. On my twenty-first birthday, my Grandmother told me that on that day she felt very happy because she was finally going to spend a week with her daughter after almost a decade of being apart. At the same time, she felt that every day during that week her heart was slowly being torn into pieces.

During that week, my parents went to the American embassy in El Salvador to apply for my sister's and my visa so that our family could be reunited in the United States. Even though both of my parents were permanent California residents, the embassy denied their request. They said that in order for them to grant this petition, my parents would have to request our visas from the United States to the Salvadorean immigration agency. And, since at the time El Salvador was still in civil war, visas were not easily granted, at least to those people who did not own property, or could not bribe the Salvadorean officials. In turn, there would be another five to eight year wait period before the Salvadorean immigration agency would make their decision. Both of my parents were greatly disappointed at this information. They thanked the Ambassador for his advice and left the building.

When my parents came from the American Embassy, I remember my mother saying, "like Hell I'm going to spend eight more years away from my children." The following day, just like our parents ten years prior, my sister and I began our three thousand mile journey to the United States in hope of a better life. Within a month period, Krissia and I had illegally crossed three international borders and were enrolled in school using fake social security

numbers, both taking classes in a completely foreign language. My mother said that if we were going to learn how to speak English, we had to “eat, sleep and dream in English”, speaking Spanish at the house was forbidden. Furthermore, we were enrolled in strictly English speaking courses. In nine months, my sister and I were able to communicate with our peers, and within two years we were getting our first B’s and A’s competing against native born English speakers. It would be another ten years, after I was already attending my first year in college that I was finally granted “true” permanent resident status. This was another battle, since I my university threatened to withdraw my enrollment because they couldn’t find a match for my proposed social security number, and because my “alien” number also did not match their records. In other words, they were kicking me out of school even though I had a 3.75 GPA. I decided to take my transcripts to the Salvadorean Embassy in Los Angeles and was determined not to leave the office until I pleaded my case to the Ambassador, I wanted to remain in school and get my bachelor’s degree. That year, while the university denied me of financial aid, I worked in construction with my father in order to pay for my books and for my tuition. Fortunately, Ambassador Don Calderon Sol, granted me a one year visa, and a promise that he would personally oversee my “green card” application. By the end of the year, I had a valid social security number and a valid spot in the university. Within five years, I was the first of my family to graduate from college, obtaining two bachelors of science degrees, one in biochemistry and the other in microbiology. While in college I successfully completed two research projects under two different advisors, one in each department, and also participated in a summer scientific research project in Palma de Mallorca, Spain. During my final year at Cal Poly, I was selected as one of two students

to represent my university in the 2002 California State Undergraduate Research Competition. That year, I applied to Cornell University and was admitted into the PhD program in biochemistry, cell and molecular biology (BMCB). Since arriving at Cornell, I have completed all of my formal graduate courses in BMCB with a minor in genetics. Besides science, my greatest passions include drinking good wines, listening music, making love, reading and studying Portuguese (not necessarily in that particular order). Thinking back, my Grandmother was always right, my parents did return to El Salvador to bring my sister and me to “the land of opportunities, where dreams do come true”.

*Para mis hijos con todo corazón*

## ACKNOWLEDGMENTS

I would like to thank my thesis advisor John Lis for being a great mentor and friend. Since I've known him, I've always felt that his door is constantly open to discuss recent successes, troubles and failures. Even when his schedule was completely booked, John always found the time to listen to my problems, or as it turns out, editing my abstract for this thesis proposal even if it meant making him "slightly" late for his next appointment. John, thank you for all your patience and guidance. I am also very grateful to the rest of my committee for providing critical feedback during my graduate career. A special thanks to Hua Shi and Marc Antonyak. Their influence has dramatically sharpened my project. Also, a special thanks to all of my friends for giving me part of their lives while in this transitory journey. Most importantly, my deepest and most sincere feelings of gratitude to my family, they are the principal source of energy that fuels my inner drive. They have provided me with the strength and willingness to undertake the hardships of graduate school, taught me the meaning of tolerance, continue to inspire me become a more patient person.

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## LIST OF ABBREVIATIONS

HSF1- Heat shock transcription factor-1

HSE- Heat shock element

iaRNA- inhibitory aptamer RNA

DBD- DNA binding domain

RNAPII- RNA Polymerase II

CTD- C-terminal domain

PIC- Pre-initiation complex

GAF- GAGA associated factor

EMSA- Electrophoretic mobility shift assay

ChIP- Chromatin immunoprecipitation assay

## **CHAPTER ONE: INTRODUCTION**

All organisms have a well-conserved heat shock response that is universal among species as diverse as bacteria, plants and animals (Lindquist, and Craig, 1988a). Upon heat stress, the heat shock transcription factor (HSF1) induces the expression of molecular chaperones that allows organisms cope with the protein damage caused by the stress. Interestingly, these molecular chaperones, or heat shock proteins, are amongst the most highly conserved proteins throughout the animal and plant kingdoms (Hendrick, and Hartl, 1993; Lindquist, 1980). Because a high concentration of heat-denatured proteins are deleterious to the cell, evolution puts strong selective pressure on living systems to elicit an efficient stress response, something that rapidly evolving systems like cancer cells utilize quite efficiently. Therefore, to further understand genetic instability disorders like cancer, and at the same time to broaden the effort to identify novel proteins that promote cellular transformation, one must first be capable of efficiently disrupting the HSF1 mediated chaperone network that maintains cancer cell survival.

### **1.1. The Heat Shock Response**

The heat shock response is one of the most robust cellular responses to external stimuli found in nature. Upon heat exposure, the general transcription and translation programs become rapidly attenuated. At the same time, heat stress induces the robust transcription of heat shock genes, and the efficient translation of the heat shock gene mRNA transcripts. In this way, living systems can quickly and efficiently synthesize heat shock proteins, or molecular chaperones that allow cells to overcome heat induced protein damage by catalyzing protein refolding reactions. The rapid kinetics of cellular

reprogramming during heat exposure emphasizes the importance of maintaining the quality of cellular proteins necessary for survival. Strikingly, there is a direct correlation between an organism's environment and the temperature at which the heat shock response is triggered. For instance, in *Drosophila melanogaster* heat shock is effectively triggered at temperatures that correlate to warm summer days, 33-37°C (Lindquist, 1980). Among thermophilic bacteria actively growing at temperatures of 50°C, the heat shock response is triggered at approximately 60°C (Daniels, McKee, and Doolittle, 1984). In arctic clams that live in temperatures near 0°C, the heat shock response is activated at around 5-10°C (Park, Ahn, and Lee, 2007). Among humans, the heat shock response is activated near fever level temperatures, ~42°C. These observations indicate that the heat shock response is regulated by intrinsic cellular factors (to be discussed later in this chapter), rather than by direct sensing of heat stress by HSF1 (Zhong, Orosz, and Wu, 1998). To date, the most convincing *in vivo* evidence that supports this hypothesis derived from studies that introduced human HSF1 protein into *Drosophila* cells or *Xenopus* oocytes depleted of their endogenous HSF1 proteins. In these experiments, the human HSF1 protein functionally complemented the loss of the endogenous HSF1, and remarkably, the human protein efficiently induced a robust heat shock response at the organisms' critical temperature (37°C for both *Drosophila* and *Xenopus*), instead of the human critical temperature of 42°C (Clos, Rabindran, *et al*, 1993; Zuo, Rungger, and Voellmy, 1995).

Lindquist and colleagues first proposed that heat shock proteins bind denatured proteins and catalyze protein-refolding reactions (Lindquist, 1980; DiDomenico, Bugaisky, and Lindquist, 1982; Pelham, 1986). Indeed, induction of an effective heat shock response provides an advantageous biological



adaptive response to external stress that promotes cell survival by refolding heat denatured proteins. In cases where the damage is too severe, heat shock proteins efficiently target heat denatured polypeptides for degradation (Freeman, and Morimoto, 1996). By definition, chaperones constitute “a family of unrelated proteins that mediate the correct assembly of other polypeptides, but are not themselves components of the final functional structure” (Ellis, and van der Vies, 1991; Hendrick, and Hartl, 1993). Intriguingly, upon exposure to elevated temperatures, chaperones become highly concentrated at the nucleus and the cytoskeleton, partly because these organelles experience the most protein damage (Welch, and Suhan, 1985; Lepock, Frey, *et al*, 2001). If the stress is too severe and the cell can’t resume its normal transcriptional program or re-establish its normal cytoskeletal structure because of extensive protein damage, these organelles send specific signals to the mitochondria to initiate apoptosis. Given the functional significance of these organelles in regulating almost every aspect of cellular homeostasis, and the strong selection pressures placed on living systems since the origins of life, it is not surprising that the heat shock response evolved to be highly sensitive to even slight changes in temperature.

## **1.2 The HSF Family is Highly Conserved Among Eukaryotes**

HSF1 activates the expression of a specific set of heat shock genes, resulting in the accumulation of molecular chaperones that allow the organism to cope with cellular damage induced by the thermal stress. Structural and biochemical studies reveal that the master regulator of heat shock gene expression, HSF1, is composed of at least three domains: a DNA binding domain (DBD), a trimerization domain (TD) and an acidic activation domain

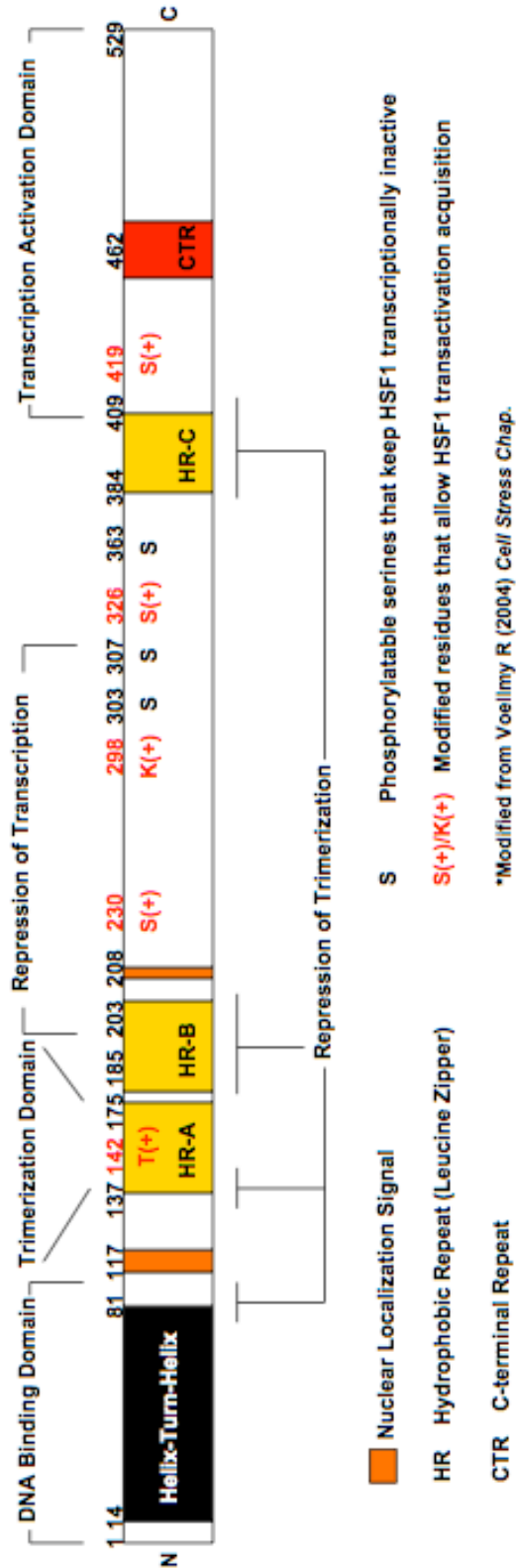
(AD) (Figure 1.1) (Green, Schuetz, *et al*, 1995; Peteranderl, and Nelson, 1992; Pirkkala, Nykanen, and Sistonen, 2001; Sorger, and Nelson, 1989; Wisniewski, Orosz, *et al*, 1996; Wisniewski, Orosz, *et al*, 1996; Zuo, Rungger, and Voellmy, 1995). Interestingly, the DBD is the most conserved HSF1 domain across all members of the HSF family among different species (Figure 1.2A) (For more detail on the conservation and structure of various HSF proteins among different eukaryotes see Pirkkala, Nykanen, and Sistonen, 2001). To date, the crystal and solution structures of *Kluyveromyces lactis* and *D. melanogaster* reveal that the HSF1 DBD is a typical helix-turn-helix domain comprised of three alpha helices, four beta strands, and a solvent exposed loop (Figure 1.2A & B) (Damberger, Pelton, *et al*, 1994; Harrison, Bohm, and Nelson, 1994; Littlefield, and Nelson, 1999; Vuister, Kim, *et al*, 1994). The HSF1 DBD third helix (H3) is the most conserved stretch of amino acids, consisting of mainly positively-charged residues that allow the molecule to make specific contacts with various phosphate groups on the major groove of heat shock gene promoters (Figure 1.2B) (Littlefield, and Nelson, 1999; Vuister, Kim, *et al*, 1994).

The HSF1 preferred DNA binding sites are called the heat shock elements (HSE), and are composed of inverted repeats containing an nGAAn consensus sequence (Fernandes, Xiao, and Lis, 1994; Fernandes, Xiao, and Lis, 1995). It has been observed that the relative affinity of HSF1 to different promoters is dependent on the specific arrangement and spacing of each HSE within promoters, and the proximity of HSF1 molecules to one another, since HSF1 binds to DNA in a cooperative manner (Amin, Fernandez, *et al*, 1994;

**Figure 1.1: Diagram of human heat shock transcription factor-1 (HSF1).**

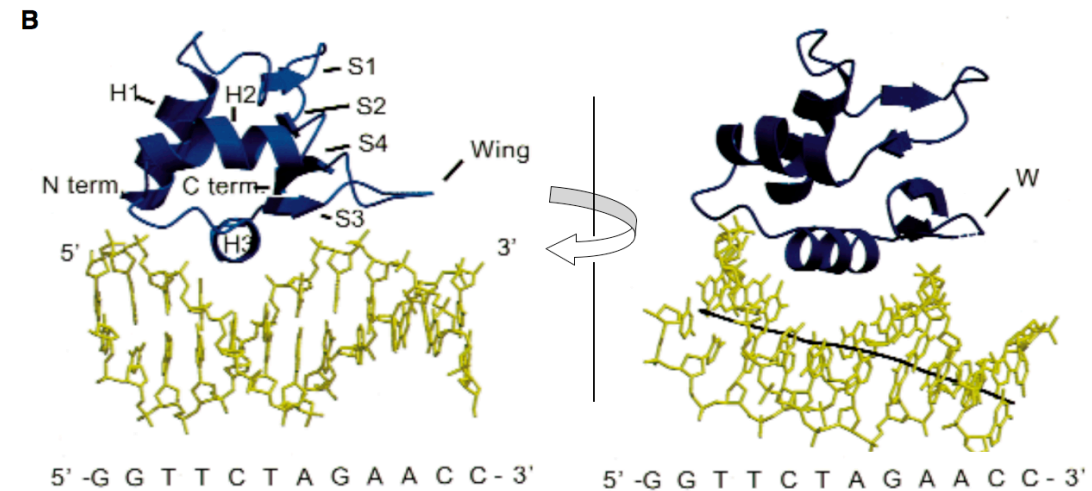
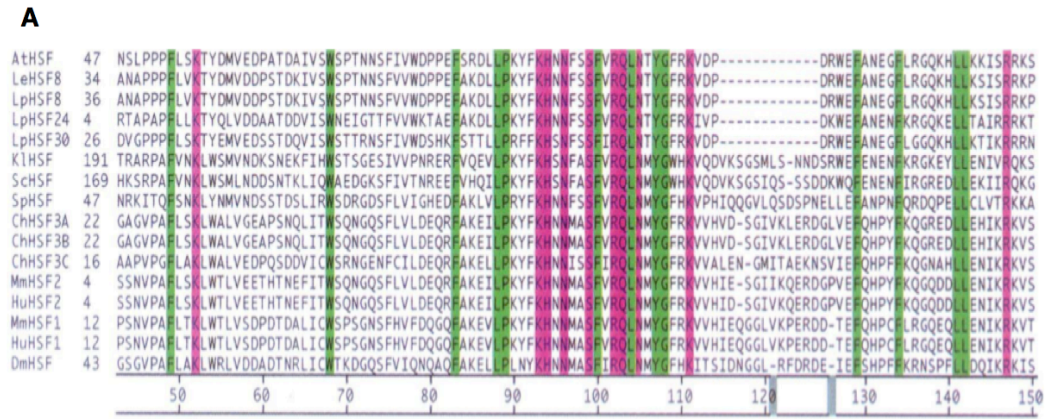
In this depiction, HSF is composed of three main domains: DNA binding domain comprised of a typical helix-turn-helix, a homo-trimerization domain containing hydrophobic repeats (leucine zippers), and a transcriptional activation domain. Amino acids labeled in black residues indicate serine phosphorylation sites that maintain HSF1 in a transcriptionally inactive state; while red-labeled residues indicate those sites that become modified to potentiate HSF1 trans-activation activity. Repression of trimerization corresponds to sites that are bound to molecular chaperones that maintain HSF1 in a transcriptionally 'inactive' monomer conformation (Modified from Voellmy R. **2004** *Cell Stress & Chaperones*).

**General Structure of Human Heat Shock Transcription Factor-1 (HSF1):**



**Figure 1.2: HSF1 has a ‘winged’ helix-turn-helix motif that is highly conserved among eukaryotes.**

(A) Amino acid sequence alignment of the DNA binding domain between different eukaryotes: *Arabidopsis thaliana* (AtHSF), tomato (LeHSF8, LpHSF8, 24,30), *Kluyveromyces lactis* (KlHSF), *Saccharomyces cerevisiae* (ScHSF), *Saccharomyces pombe* (SpHSF), chicken (ChHSF), *Mus musculus* (MmHSF1, 2), human (HuHSF1, 2) and *Drosophila melanogaster* (DmHSF) shows that the DBD is highly conserved, particularly in the DNA contact region, helix 3 (H3) (Modified from Vuister G.W. et. al. **1994** *Nature*). (B) The 1.75Å crystal structure of *Kluyveromyces lactis* HSF-DBD complexed to DNA shows that the DBD is a typical ‘winged’ helix-turn-helix structure. In this illustration, helix-3 recognizes and binds to the major groove of DNA that contains an NGAAN consensus sequence (Modified from Littlefield O and Nelson HC **1999** *Nature*).



Xiao, Perisic, and Lis, 1991). In humans, it has been found that HSF1 and HSF2 have specific preferences for particular HSE arrangements on gene promoters: HSF1 prefers an array of 4-5 units of nGAAn pentameric consensus sequences, while HSF2 prefers 2-3 repeating units (Liu, Liu, *et al*, 1997). Therefore, it is likely that the observed developmental differences associated with *HSF1*<sup>-/-</sup> or *HSF2*<sup>-/-</sup> knock-out mice are due to differences in gene expression and the HSE composition of specific gene promoters that are regulated by each molecule (see section 1.6).

The HSF1 trimerization domain consists of hydrophobic heptad repeats (HR-A/B & C) made of a series of helical coiled-coil domains known as 'leucine zippers'. In the absence of stress, HSF1 is maintained in a transcriptionally inactive state by associations with multi-chaperone complexes and the hydrophobic repeats (Figure 1.1) (Green, Schuetz, *et al*, 1995; Pirkkala, Nykanen, and Sistonen, 2001; Wisniewski, Orosz, *et al*, 1996; Rabindran, Haroun, *et al*, 1993; Zuo, Baler, *et al*, 1994). At the same time, the hydrophobic repeats allow HSF1 to become a stable homotrimer after an initial heat shock exposure (Figure 1.1) (Sorger, and Nelson, 1989). The HSF1 acidic activation domain (AD) is located at the C-terminus and contains several acidic and hydrophobic residues that associate with the transcriptional apparatus to induce heat shock gene expression (Figure 1.1, CTR) (Wisniewski, Orosz, *et al*, 1996). Currently, strong evidence suggests that heat shock induces major conformational changes within the activation domain resulting in a dramatic increase in the AD helical content (Bulman, and Nelson, 2005; Pattaramanon, Sangha, and Gafni, 2007). It is hypothesized that these newly formed  $\alpha$ -helices give the activation domain more rigidity, and allow

HSF1 to make specific contacts with proteins that modify the transcriptional machinery that results in elevated heat shock gene expression.

### 1.3. Heat Shock Transcription Factor Proteins

In bacteria, the *rpoH* gene product (heat shock sigma factor- $\sigma^{32}$ ), is responsible for inducing the heat shock response. Under regular growth conditions,  $\sigma^{32}$  is maintained at low concentrations due to its rapid protein turnover; however during stress conditions,  $\sigma^{32}$  concentrations increase rapidly by becoming part of the transcriptional apparatus that up-regulates its own expression levels as well as the expression of other heat shock genes (Grossman, Erickson, and Gross, 1984; Straus, Walter, and Gross, 1987). Among eukaryotes, the transcription factors that activate heat shock gene expression are called heat shock transcription factors (HSFs).

*Saccharomyces cerevisiae* and *Drosophila melanogaster* express a single HSF1 protein that responds to a variety of other stress conditions including damage by reactive oxygen species and exposure to heavy metals (Wiederrecht, Seto, and Parker, 1988; Sorger, and Nelson, 1989; Clos, Westwood, *et al*, 1990).

Plants and vertebrates have evolved multiple HSF isoforms that appear to have specialized functions (Czarnecka-Verner, Yuan, *et al*, 2000; Nakai, and Morimoto, 1993; Nakai, Tanabe, *et al*, 1997; Rabindran, Giorgi, *et al*, 1991; Sarge, Zimarino, *et al*, 1991; Schuetz, Gallo, *et al*, 1991; Xiao, Zuo, *et al*, 1999). Strikingly, plants display the largest diversity of heat shock factors among all groups of organisms. For example, *Arabidopsis thaliana* and *Lycopersicum esculentum* (tomato) express over 20 different HSF isoforms, each displaying distinct modes of regulation and activation (Czarnecka-Verner,



Yuan, *et al*, 2000; Czarnecka-Verner, Pan, *et al*, 2004; Nover, Bharti, *et al*, 2001). Vertebrates contain four HSF family members, three of which are found among mammals, HSF1, HSF2, and HSF4. The fourth member, HSF3, is avian specific (Tanabe, Kawazoe, *et al*, 1998; Pirkkala, Nykanen, and Sistonen, 2001).

In *S. cerevisiae*, HSF1 is essential for cellular viability and for vegetative growth (Wiederrecht, Seto, and Parker, 1988). However, unlike yeast, animals do not require HSF1 activity for general cell growth; rather, HSF1 is required during specific developmental stages. For instance, in *Drosophila* HSF1 activity is required only during early larval development and during oogenesis (Jedlicka, Mortin, and Wu, 1997), while in *C. elegans* HSF1 is required for longevity (Morley, and Morimoto, 2004; Walker, Thompson, *et al*, 2003). Among mammals, HSF1 is the transcription factor responsible for inducing the expression of molecular chaperones in response to a wide variety of stresses, including heat (Xiao, Zuo, *et al*, 1999; Sarge, Zimarino, *et al*, 1991; Murray, Whitfield, *et al*, 2004). Intriguingly, HSF1's cytoprotective role during stress cannot be substituted by any other members of the HSF family (Xiao, Zuo, *et al*, 1999; McMillan, Christians, *et al*, 2002).

Recently, HSF1 has been implicated as a “non-classical oncogene” because its activity promotes tumor formation and maintenance of the transformed phenotype in cancer cells, without affecting the viability of normal cells (Dai, Whitesell, *et al*, 2007). Therefore, it seems that HSF1 functions to promote cell survival even under conditions that could potentially become deleterious to cells, such as development of the transformed state.

Unlike HSF1, HSF2 does not regulate the expression of heat shock proteins (Kallio, Chang, *et al*, 2002; McMillan, Christians, *et al*, 2002; Wang,

Zhang, *et al*, 2003). Instead, HSF2 is required specifically during mammalian embryogenesis and brain development (Kallio, Chang, *et al*, 2002; McMillan, Christians, *et al*, 2002; Paslaru, Morange, and Mezger, 2003). The mammalian HSF4, although similar in structure to both HSF1 and HSF2, lacks an acidic activation domain (Pirkkala, Nykanen, and Sistonen, 2001). Interestingly, HSF4 is highly abundant within the eye tissues of mammals, and is apparently required for maintaining the integrity of the eye-lens and preventing cataract formation (Fujimoto, Izu, *et al*, 2004). Because HSF4 does not contain an activation domain, it is currently believed that HSF4 is involved in attenuating heat shock gene expression by competing with HSF1 for binding to heat shock elements (Nakai, Tanabe, *et al*, 1997). Amongst birds, HSF3 has been found to work in concert with HSF1 to properly induce heat shock gene expression during heat stress (Nakai, and Morimoto, 1993; Tanabe, Kawazoe, *et al*, 1998).

#### **1.4. HSF1 Controls Transcription of Heat Shock Genes at Multiple Levels**

The heat shock genes, as their name implies, were first identified in the 1960's in *D. melanogaster* by visualizing the decondensation of specific bands in the polytene chromosomes during temperature elevation {reviewed in (Ritossa, 1996)}. The underlying mechanism for this 'puffing' response is due to the decondensation of chromatin by the transcriptional apparatus at these highly induced loci (i.e, heat shock genes) (Jedlicka, Mortin, and Wu, 1997; Petesch, and Lis, 2008; Ritossa, 1996). Over the last two decades, our group has focused in understanding the molecular mechanism of HSF1 mediated

gene expression using the *Drosophila Hsp70* gene as a model (Lis, 1998; Saunders, Core, and Lis, 2006).

Strikingly, immediately after cells are exposed to elevated temperatures, HSF1 becomes rapidly activated and transitions from an globular-monomeric form into a rigid-homotrimer form that binds heat shock gene promoters in a highly cooperative manner (Sorger, and Nelson, 1989; Westwood, and Wu, 1993; Xiao, Perisic, and Lis, 1991; Fernandes, Xiao, and Lis, 1994; Zuo, Baler, Dahl, and Voellmy, (1994); Fernandes, Xiao, and Lis, 1995). After the first few minutes following heat stress, HSF1 saturates the promoters of heat shock genes and remains at these sites throughout the duration of the heat shock response (Boehm, Saunders, *et al*, 2003). Such HSF1 binding to heat shock promoters induces a highly orchestrated series of molecular events that results in over one hundred-fold gene expression (Lis, 1998; Saunders, Core, and Lis, 2006).

One major rate-limiting step that HSF1 must overcome in order to induce a robust heat shock response is the effective recognition of its cognate sites on DNA. Under normal growth conditions, the GAGA Associated factor (GAF) is responsible for maintaining the *Hsp70* promoter in a nucleosome-free 'open' conformation that is feature is critical for effective HSF1 binding during heat stress (Figure 1.3A) (Shopland, Hirayoshi, *et al*, 1995; Tsukiyama, and Wu, 1995; Wilkins, and Lis, 1997). Shopland *et al.* used various methods to demonstrate that GAF is also critical for the establishment of the pre-initiation complex (PIC), and the formation of a transcriptionally engaged yet paused RNA Pol II (Shopland, Hirayoshi, *et al*, 1995).

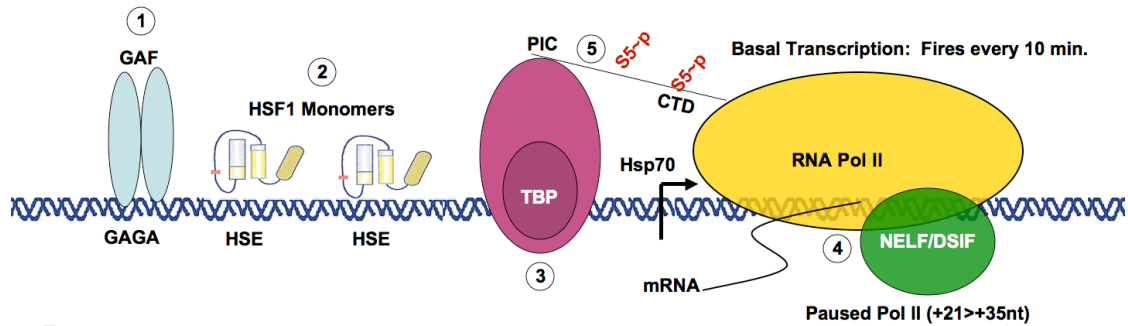
#### **1.4.1 HSF1 facilitates RNA Pol II release from PIC's**

Among eukaryotes, numerous studies have proposed that acidic activators participate in the formation of a pre-initiation complex (PIC) through the recruitment of TATA binding protein (TBP) (Xiao, Friesen, and Lis, 1995; Bhaumik, Raha, *et al*, 2004; Larschan, and Winston, 2005; Park, Werner, *et al*, 2001). This mechanism has support in *S. cerevisiae* because under normal growth conditions HSF1 occupies the promoters of many heat shock genes at high levels to facilitate the establishment of a functional PIC. In flies, heat shock genes contain low amounts of HSF1 under normal growth conditions, a pre-assembled PIC, and a promoter in an 'open' conformation. It has been speculated that among higher eukaryotes, HSF1 competes with transcription factors that bind and maintain RNA Pol II molecules at the core promoter, thus facilitating RNA Pol II promoter escape (Mason, and Lis, 1997).

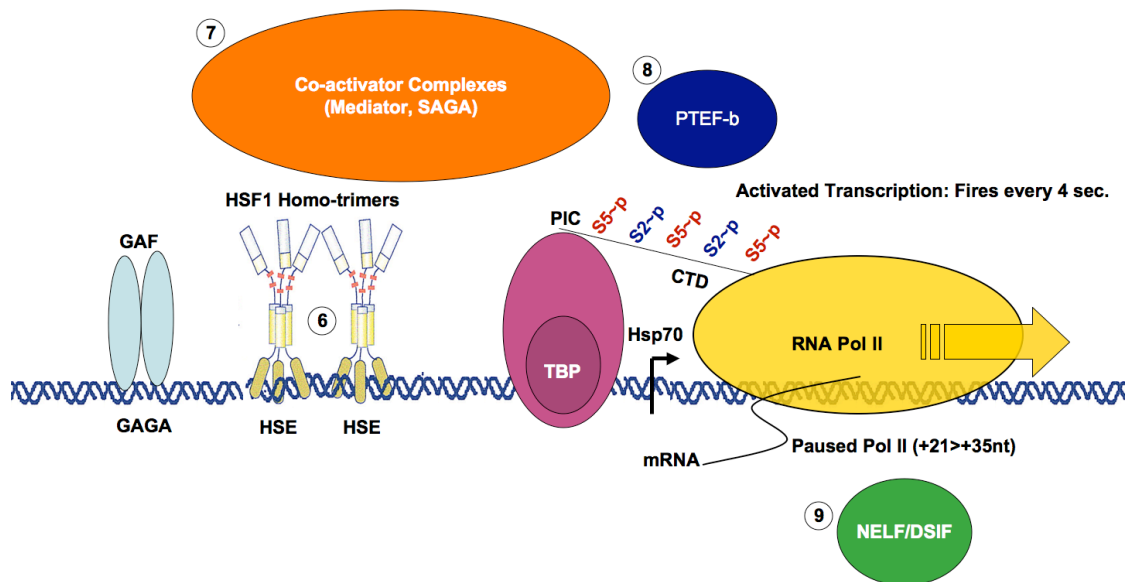
Studies in *Drosophila* and mammalian cells have shown that acidic activators like VP16, E2F1, p53, and HSF1 can all stimulate promoter escape by competing with the physical interactions between RNA Pol II and transcription factors at the core promoter, specifically TBP and TFIIB (Yuan, and Gurley, 2000; Blau, Xiao, *et al*, 1996; Mason, and Lis, 1997; Kadonaga, 2004; Yankulov, Blau, *et al*, 1994). Not only is it likely that HSF1 affects efficient promoter escape, HSF1 may also participate in subsequent steps of the transcription cycle such as the establishment of a paused RNA polymerase, the recruitment of co-activator complexes, recruitment of proteins kinases that modify the transcriptional machinery, and the overall maintenance of RNA Pol II molecules at heat inducible genes (Saunders, Core, and Lis, 2006).

**Figure 1.3: Proposed role of HSF1 under ‘normal’ (NHS) conditions and during heat shock conditions. (A).** Under normal non-heat shock conditions (NHS), the *Drosophila Hsp70* promoter is maintained in a nucleosome free ‘open’ conformation by GAF (1). Under these conditions, HSF1 monomers (2) bind with rapid kinetics to the heat shock elements (HSE) binding sites. Together with the pre-initiation complex (3), HSF1 helps establish a transcriptionally engaged, yet paused RNAP II (4) 20-40 base pairs from the start site, an association that is stabilized by the NELF/DSIF repressor complex (4). Under these conditions (NHS), basal transcriptional machinery fires once every ten minutes. **(B).** Upon heat shock treatment, HSF1 is activated, resulting in homo-trimerization and stable binding to heat shock gene promoters (6). Following HSF1 binding to DNA, the molecule acquires trans-activation competence by hyper-phosphorylation (6), resulting in the recruitment of co-activator complexes (i.e. Mediator or SAGA/CBP)(7), and the recruitment of protein kinases (8) that modify the CTD-RNAPII domain into an transcriptionally elongating competent form (i.e. PTEF-b), along with the release of negative elongation factors NELF (9). Under these conditions, RNAPII pausing still occurs at +21 and +35 nt, yet RNAPII is released once every four seconds.

**A. Non-Heat Shock 'Normal' conditions (NHS)**



**B. Heat Shock Treatment (HS)**



#### **1.4.2. HSF1 promotes promoter escape during heat shock**

During non-heat shock conditions heat shock genes contain a transcriptionally active RNA polymerase, yet RNA Pol II is known to pause at positions near +21 and +35 from the *Hsp70* transcription start site (Figure 1.3A) (Rougvie, and Lis, 1988; Xiao, and Lis, 1989; O'Brien, and Lis, 1991; Rasmussen, and Lis, 1993; Core, Waterfall, and Lis, 2008). Intriguingly, the two peaks of RNA Pol II pausing (+21 and +35) are separated by 1 turn of the DNA helix, suggesting that the transcriptionally engaged RNA Pol II molecules interact with factors also associated with one side of the DNA helix that inhibit effective transcription elongation, an impediment that is relieved during heat stress. Under non-heat shock conditions, the repressor complex **NELF/DSIF/Spt4-Spt5** promotes RNAP Pol II pausing at heat shock loci in *Drosophila* and human cells (Wu, Yamaguchi, *et al*, 2003). Upon heat shock treatment, the NELF subunit is released from the *Hsp70* promoter, and this release coincides with the subsequent recruitment of transcription elongation factors (DSIF, Spt4-6, PTEFb), RNA capping and processing factors which modify the transcriptional apparatus into a mature transcriptionally elongating form (Lindstrom, Squazzo, *et al*, 2003; Saunders, Core, and Lis, 2006). Given the close proximity of NELF/DSIF/Spt4-Spt5 repressor complex with HSF1 binding sites, it is likely that HSF1 binding to heat shock promoters induces NELF disassembly immediately following heat shock induction through the recruitment and kinase activity of PTEFb (Figure 1.3B).

### **1.4.3. HSF1 recruitment of co-activators & chromatin remodeling machines to heat shock genes upon stress**

Robust heat shock gene expression requires the recruitment of co-activator complexes that bridge interactions between HSF1 and the transcriptional apparatus. The Mediator complex is a large multi-subunit complex that was originally identified as a yeast extract that induced basal and activated transcription *in vitro* (Kim, Bjorklund, *et al*, 1994). Soon after its initial discovery, this complex was found to stimulate transcription from a wide variety of acidic activators by bridging contacts between the activators and the transcriptional apparatus (Conaway, Sato, *et al*, 2005; Fan, Chou, and Struhl, 2006; Kim, Kwon, *et al*, 2004; Kim, and Lis, 2005; Kornberg, 2005; Lee, Kim, and Lis, 1999; Park, Werner, *et al*, 2001; Singh, Erkine, *et al*, 2006). During heat shock treatment, HSF1 rapidly recruits the Mediator complex to heat shock genes (Figure 1.3B) (Park, Werner, *et al*, 2001). Intriguingly, the rapid recruitment of Mediator complexes to heat shock genes depends on HSF1 activity rather than the status of the transcriptional machinery RNA Pol II, indicating that Mediator recruitment precedes the establishment of an elongation-competent transcriptional apparatus. This observation also directly questioned the 'holoenzyme' hypothesis, which argues that acidic activators recruit pre-assembled Mediator-RNA Pol II complexes. The fact that Mediator complex remains bound to promoters during active transcription strongly suggests that the assembly of the transcriptional apparatus occurs in a stepwise manner (Kornberg, 2005). In *Drosophila*, it has been demonstrated that HSF1 interacts directly with Mediator by making contacts with the Med17, Med23, and Med25 subunits (Kim, Kwon, *et al*, 2004; Kim and Lis, 2005).



Because virtually all RNA Pol II transcribed genes require Mediator for basal or induced expression (Holstege, Jennings, *et al*, 1998; Kornberg, 2005), it is likely that HSF1 trans-activation activity at heat shock genes occurs through its associations with this large (~1MDa) multi-subunit complex.

Acidic activators can influence gene expression by modifying the chromatin landscape of target genes via recruitment of nucleosome remodeling complexes (Swi/Snf) (Brown, Imbalzano, and Kingston, 1996; Yudkovsky, Logie, *et al*, 1999), the histone acetyl-transferase Spt5-Ada-Gcn5 (SAGA) complex (Gregory, Schmid, *et al*, 1999; Larschan, and Winston, 2005; Lemieux, and Gaudreau, 2004; Zanton, and Pugh, 2004; Zanton, and Pugh, 2006), and nucleosome methyl-transferases or ubiquitinases (Sala, La Rocca, *et al*, 2008; Shukla, Chaurasia, and Bhaumik, 2008; Smith, Petruk, *et al*, 2004). Among yeast, heat shock results in the disassembly of the TFIID pre-initiation complex at genes that are repressed by the stress; however, genes that are upregulated recruit high levels of the SAGA complex (Zanton, and Pugh, 2004; Zanton, and Pugh, 2006). It has been recently demonstrated that acidic activators recruit the multi-subunit SAGA complex through the SAGA-Tra1 subunit (Bhaumik, Raha, *et al*, 2004). Intriguingly, biochemical and genetic analyses demonstrate that SAGA recruitment precedes Mediator binding to stress-inducible genes. It is likely that a similar mechanism exists in *Drosophila* given that, under non-inducing conditions, the *Hsp70* gene is associated with significant amounts of the SAGA homolog TRRAP/GCN5 and the Mediator complex (Lebedeva, Nabirochkina, *et al*, 2005; Smith, Petruk, *et al*, 2004; Kim, Kwon, *et al*, 2004). Furthermore, the TRRAP/GCN5 and Mediator complexes, like their mammalian counterparts, are dramatically recruited to heat shock genes by thermal stress (Lebedeva, Nabirochkina, *et*

*al*, 2005). Collectively, these studies strongly suggest that these co-activator complexes are highly responsive to varied levels of HSF1 activity (Figure 1.3B).

Recently, Petesh and colleagues demonstrated that poly-ADP ribose polymerase (PARP) is a critical modulator of the chromatin landscape at *Hsp70* during heat stress (Petesch, and Lis, 2008). This novel role for PARP is likely due to its ability to modify, and therefore inhibit, proteins that maintain nucleosomes in a compact state such as histone H1 (Krishnakumar, Gamble, *et al*, 2008; Sala, La Rocca, *et al*, 2008). It is likely that HSF1 recruits and activates PARP, the two proteins are in close proximity to transcription start sites, and upon heat shock, PARP becomes 'activated' and redistributes over the entire locus. It has been suggested that PARP facilitates RNA Pol II mediated gene expression by traveling along nucleosomes and exerting its anti-repressive effects on chromatin (Petesh and colleagues personal communication). In mammals, the *Hsp70* locus is modified by the activity of the Swi/Snf remodeling complex during heat stress (Brown, Imbalzano, and Kingston, 1996); however, this particular chromatin remodeling complex does not seem to regulate the expression of *Drosophila Hsp70* (Armstrong, Papoulas, *et al*, 2002).

#### **1.4.4. Establishment of an elongation competent transcriptional apparatus**

Establishment of a robust transcriptional response to heat stress exposure does not occur until the largest RNA Pol II subunit, Rpb1, is hyper-phosphorylated at specific serine residues. Hyper-phosphorylation of the C-terminal domain (CTD) of Rpb1 is an active process that only occurs after heat

shock exposure (Figure 1.3B) (Saunders, Core, and Lis, 2006). The precise mechanism by which activators contribute in this recruitment process is still a mystery; however, given the potent recruitment functions of acidic activators, it is likely that HSF1 plays essential roles for the recruitment of CTD kinases including TFIIH and PTEFb (Ni, Schwartz, *et al*, 2004; Schwartz, Larochelle, *et al*, 2003). Currently, strong *in vivo* evidence suggests that the effective recruitment of PTEFb to heat shock genes depends largely on the activities of HSF1 and the presence of a transcriptionally engaged yet paused RNA Pol II (Figure 1.3B) (Lis, Mason, *et al*, 2000). Whether a direct physical interaction between HSF1 and the CTD kinases exists is still to be determined. It is tempting to speculate that HSF1 promotes the establishment of an elongation competent transcriptional apparatus by modulating the activity of TF-IIB (Chen, and Hampsey, 2004; Lin, Ha, *et al*, 1991; Roberts, and Green, 1994; Tubon, Tansey, and Herr, 2004), the recruitment of TFIIS (Adelman, Marr, *et al*, 2005), Spt6 (Kaplan, Laprade, and Winston, 2003), or FACT (Saunders, Werner, *et al*, 2003) given that these proteins affect the intensity of heat shock induction.

Dissecting the role of HSF1 on the general transcriptional machinery has been a difficult task because of the extremely rapid kinetics during heat shock. To gain a better understanding on HSF1 role, future studies should try to address fundamental questions regarding the role of HSF1 activity on co-activator interactions, and their effects on the transcriptional apparatus using HSF1 specific inhibitors. For instance, is HSF1 activity necessary for the maintenance of co-activator interactions at promoter regions once transcription has already been established? How stable are these interactions? Which transcription factors fail to be recruited if HSF1 activity is inhibited before or

after heat shock induction? Does HSF1 inhibition affect the recruitment of new RNA Pol II molecules during heat-induced conditions? How is the chromatin landscape affected by HSF1 inhibition? Are chromatin remodeling complexes, PARP, or elongation factors affected by HSF1 inhibition? Are specific HSE's necessary for expressing *Hsp70* during basal or activated transcription? These questions could potentially be addressed by using small molecule inhibitors that modulate the heat shock response or by generating novel HSF1 modulators that will be discussed in subsequent sections in this chapter. Because HSF1 is central to heat shock gene expression, and therefore, the general maintenance of proteome quality control, it is essential to perform more rigorous studies aimed at dissecting the molecular basis of HSF1-mediated gene activation to gain a better understanding of diseases like cancer, neurodegenerative disorders, and natural processes like aging (Soti, and Csermely, 2003).

### **1.5. Multiple Levels of HSF1 Regulation**

HSF1 is regulated at the levels of oligomerization, DNA binding, phosphorylation, acquisition of trans-activation competence, sub-cellular localization, and co-factor interactions (Voellmy, 2004). As mentioned previously, nearly all metazoans have the same general HSF1 architecture, with a DNA binding domain close at the N-terminus, a trimerization domain consisting of a series of leucine zippers (Peteranderl, and Nelson, 1992; Sorger, and Nelson, 1989), and a trans-activation domain close to the C-terminus (Figure 1.1) (Green, Schuetz, *et al*, 1995; Shi, Kroeger, and Morimoto, 1995; Wisniewski, Orosz, *et al*, 1996; Zuo, Rungger, and Voellmy, 1995). The first evidence that HSF1 activity was regulated at the protein level

came from findings where HSF1 was found to undergo structural changes during heat stress, resulting in a dramatic increase in molecular weight (Westwood, and Wu, 1993). Later, using a wide collection of HSF1 mutants, the Voellmy group showed that HSF1 homo-trimerization precedes potent DNA binding potential (Zuo, Baler, *et al*, 1994). Indeed, during heat shock conditions, HSF1 is rapidly activated, and transitions from a globular monomer to a rigid homo-trimer structure capable of binding heat shock promoter DNA with high affinity. Previous studies by Xiao *et al*. revealed that HSF1 binds to heat shock elements with high affinity and in a co-operative manner (Xiao, Perisic, and Lis, 1991). It is likely that once activated, the relative strength of the HSF1-DNA interaction, is perhaps one of the strongest protein-DNA interactions found in nature. Elegant structural analysis has revealed that the 'wing' motif within the HSF1 DNA binding domain significantly contributes to the cooperative DNA potential of the molecule (Figure 1.2) (Littlefield, and Nelson, 1999).

Due to the rapidity with which trimeric HSF1 binds to DNA (Boehm, Saunders, *et al*, 2003), the complete dissection of the early HSF1 induced transcriptional events has remained elusive. Recent *in vivo* imaging studies have demonstrated in real time the potent DNA binding capacity acquired by HSF1 whenever cells are shifted from non-induced conditions to 37°C (Yao, Munson, *et al*, 2006). During this transition, the dynamic association of HSF1 to DNA under non-heat shock conditions becomes highly stable at the elevated temperature. Moreover, HSF1 is rapidly redistributed from non-specific sites on nuclei to specific heat inducible loci within the first few seconds after heat exposure (Westwood, Clos, and Wu, 1991; Yao, Munson, *et al*, 2006). Collectively, these experiments suggest that HSF1 transcriptional

activation is a stepwise, regulated process that is intimately intertwined with the activities of stress-sensing molecular chaperones.

Susan Lindquist first proposed that one of the many functions of molecular chaperones is to inhibit HSF1 activity through a direct negative feedback mechanism during normal growth conditions (Lindquist, 1980). In this mechanism, cells can directly 'sense' heat-induced protein damage because heat denatured polypeptides can effectively titrate away molecular chaperones that normally maintain HSF1 in a monomeric "inactive" state (Lindquist, 1980; DiDomenico, Bugaisky, and Lindquist, 1982). Strong evidence for this hypothesis was first provided by experiments that induced protein damage in the absence of heat stress through the addition of metal ions to the cell, or the direct microinjection of heat denatured proteins into frog oocytes (Ananthan, Goldberg, and Voellmy, 1986). Together, these experiments demonstrated that artificially increasing cellular damage encouraged denatured proteins to sequester away those chaperones that inhibit HSF1 activity.

A more precise HSF1 regulatory mechanism was proposed after initial studies on the role of molecular chaperones on glucocorticoid receptors (Nadeau, Das, and Walsh, 1993; Nair, Toran, *et al*, 1996). In this regard, a similar Hsp90/p23/immunophilin complex that regulates GRs was also found to associate with the HSF1 hydrophobic regulatory regions (HR A-C, see Figure 1.1), and maintain HSF1 in a monomeric state (Zou, Salminen, *et al*, 1998; Guo, Guettouche, *et al*, 2001). This inhibitory role of Hsp90 multi-chaperone complex on HSF1 explains the observations where Hsp90 inhibition by geldanamycin induces HSF1 homo-trimerization and trans-activation, Hsp90 immunodepletion from whole cell extracts results in HSF1

homo-trimerization and DNA binding acquisition, and Hsp90 over-expression effectively attenuates the heat shock response (Zou, Guo, *et al*, 1998; Guo, Guettouche, *et al*, 2001; Knowlton, and Sun, 2001). In *Drosophila*, depletion of Hsp90/Hsp40/Hsp70 proteins by RNAi significantly enhances HSF1 trimerization, induces HSF1 binding to DNA and promotes HSF1 transcription activation (Marchler, and Wu, 2001). Similarly, Hsp70 binds to the HSF1 activation domain and directly inhibits HSF1 transcription activation activity or 'trans-activation' (Abravaya, Myers, *et al*, 1992; Shi, Mosser, and Morimoto, 1998). Moreover, proteins that regulate Hsp70 or Hsp90 can also modulate HSF1 transcription activation activity (Chen, *et al*, 1998; Dai, Zhang, *et al*, 2003; Satyal).

Acquisition of HSF1 trans-activation potential is an HSF1 regulated process first identified from studies using anti-inflammatory drugs like sodium salicylate. In this regard, addition of sodium salicylate to eukaryotic cells induces HSF1 homo-trimerization, and HSF1 binding to DNA in a transcriptionally inactive form (Jurivich, Sistonen, *et al*, 1992; Westwood, and Wu, 1993). It has been proposed that during heat shock, specific chaperone complexes modify HSF1 into becoming transcriptionally competent (Guo, Guettouche, *et al*, 2001). Dai and colleagues found that Chip, a negative regulator of Hsp70 activity, associates with HSF1 during stress conditions to stimulate HSF1 trans-activation activity (Dai, Zhang, *et al*, 2003). Moreover, under non-inducing conditions, cells that over-express Chip have increased HSF1 activity, while Chip depletion results in compromised HSF1 trans-activation activity. It is likely that by regulating acquisition of HSF1 trans-activation, cells can tailor the intensity of stress response relative to the amount of protein damage induced by the stress. Once heat-denatured

proteins are properly folded, molecular chaperones can once again bind to HSF1 homo-trimers and attenuate the stress response (Voellmy, 2004).

Like most proteins, HSF1 activity is regulated by post-translational modifications after exposure to elevated temperatures (Larson, Schuetz, and Kingston, 1988; Sorger, and Pelham, 1988), and this results in slower HSF1 electrophoretic mobility due to stress-inducible hyper-phosphorylation (Figure 1.1). Under non-inducing conditions, HSF1 is phosphorylated at Ser303, Ser307 and Ser363, and these specific modifications are known to promote HSF1 interactions with negative regulators such as Hsp70/Hsp40 complexes. During heat-shock conditions, HSF1 is hyper-phosphorylated on Ser230 and Thr142, and these particular modifications allow HSF1 to acquire trans-activation competence (Holmberg, Hietakangas, *et al*, 2001; Holmberg, Tran, *et al*, 2002). Intriguingly, under conditions where HSF1 is bound to DNA but exists in a transcriptionally inactive form, such as sodium salicylate treatment, Ser230 does not become phosphorylated. To further understand this particular modification, Sistonen and colleagues found that expression of a single Ser<sup>230A</sup> HSF1 point mutant into *HSF1*<sup>-/-</sup> mammalian cells results in the induction of 'mild' heat shock response whenever the cells are exposed to elevated temperatures (Holmberg, Hietakangas, *et al*, 2001), suggesting that other residues control the acquisition of HSF1 trans-activation competence. Recently, Voellmy and colleagues performed a systematic analysis of the phosphorylation status of HSF1 by mutating every serine, threonine or tyrosine to alanine, and these various point mutants were introduced into *HSF1*<sup>-/-</sup> cells to determine their ability to induce a heat shock response. These experiments revealed that phosphorylation at Ser<sup>326A</sup> is critical for achieving HSF1 trans-activation competence, while mutations at Ser<sup>121A</sup>, Ser<sup>230A</sup>, Ser<sup>292A</sup>, Ser<sup>303A</sup>,



Ser<sup>307A</sup>, Ser<sup>314A</sup>, Ser<sup>319A</sup>, Ser<sup>326A</sup>, Ser<sup>344A</sup>, Ser<sup>363A</sup>, Ser<sup>419A</sup>, and Ser<sup>444A</sup> are not as critical and result in a mild to moderate heat shock response (Guettouche, Boellmann, *et al*, 2005). In a separate study, Kim and colleagues found that an HSF1 mutant harboring a Ser<sup>419A</sup> substitution prevents HSF1 from entering the nucleus following heat stress, resulting in loss of a heat shock gene induction (Kim, Yoon, *et al*, 2005). Given that HSF1 is highly conserved among metazoans, it is likely that similar HSF1 regulatory systems exist in other eukaryotes.

## 1.6. Putative Functions of HSF1 in Mammalians

It is widely accepted that genetic background greatly contributes to the phenotypic variability observed in mouse knockout studies, even within the same allele (Wolfer, Crusio, and Lipp, 2002). Unlike yeast and *Drosophila*, which have a single HSF1 gene, vertebrates have multiple HSF isoforms that could potentially contribute to this phenotypic variability. Our current understanding of the *in vivo* roles of each of these transcription factors arrives from studies using HSF knockout models. The first HSF1 knockout animals contained a drug resistant cassette within the *Hsf1*<sup>-/-</sup> loci (Xiao, Zuo, *et al*, 1999). Strikingly, *Hsf1*<sup>-/-</sup> animals are viable; however these animals show severe developmental abnormalities, reduced size, an inability to induce heat shock genes or tolerate elevated heat conditions, a loss of thermotolerance acquisition, female infertility, and embryonic lethality. Such developmental abnormalities can be compared with HSF1 defects in flies, in which *Hsf*<sup>4</sup> mutants show lethality during early stages of animal development, and females are infertile due to defects in oogenesis (Jedlicka, Mortin, and Wu, 1997). Interestingly, the observed maternal effect in *Hsf*<sup>4</sup> flies or *Hsf1*<sup>-/-</sup> mice,

does not correlate with decreased levels of heat shock proteins (Christians, Davis, *et al*, 2000; Jedlicka, Mortin, and Wu, 1997). Rather, these defects occur prior to the onset of zygotic gene expression, indicating that HSF1 has alternative functions besides regulating heat shock gene expression during animal development.

The role of HSF1 during non-inducing conditions has been a topic of considerable debate. In this regard, several studies demonstrate that HSF1 regulates the basal heat shock gene expression (Murray, Whitfield, *et al*, 2004; Trinklein, Murray, *et al*, 2004); while other studies propose that it does not (Inouye, Katsuki, *et al*, 2003; Zhang, Huang, *et al*, 2002). It is likely that such discrepancies are due to differences in culturing conditions or genetic background. Sistonen and colleagues argue that among mammals HSF2 facilitates HSF1 mediated gene expression (Ostling, Bjork, *et al*, 2007). However, given that *Hsf2*<sup>-/-</sup> mouse cells do not contain reduced Hsp levels under non-inducing conditions (Wang, Zhang, *et al*, 2003), nor are compromised for eliciting a heat shock response, it is likely that HSF2 is not a major regulator of heat shock gene transcription (Kallio, Chang, *et al*, 2002; McMillan, Christians, *et al*, 2002; Wang, Zhang, *et al*, 2003). Instead, *Hsf2*<sup>-/-</sup> animals display severe fertility defects and large brain abnormalities while *Hsf2*<sup>-/-</sup> cells undergo heat-shock induction at lower temperature conditions (Kallio, Chang, *et al*, 2002; McMillan, Christians, *et al*, 2002; Paslaru, Morange, and Mezger, 2003; Wang, Zhang, *et al*, 2003). The fact the *Hsf2*<sup>-/-</sup> cells show a heat shock response at lower temperatures may be due to the fact that *Hsf2*<sup>-/-</sup> cells are already experiencing a form of stress as shown by Wang and colleagues. To date, no studies have been done to determine the role of HSF2 in tumor formation.

It is well established that *HSF1*<sup>-/-</sup> cells cannot induce Hsp25/27, Hsp60, Hsp70, Hsc70, Hsp90a & Hsp90b after heat stress exposure, making HSF1 the primary candidate capable of inducing chaperones during stress (Inouye, Katsuki, *et al*, 2003; Zhang, Huang, *et al*, 2002; Xiao, Zuo, *et al*, 1999). Unlike unicellular organisms where HSF1 controls the expression of a large chaperone network in response to different forms of stresses (Eastmond, and Nelson, 2006), mammalian HSF1 up-regulates only few subsets of genes that are specific to each type of stress condition (Murray, Whitfield, *et al*, 2004; Trinklein, Murray, *et al*, 2004). It is possible that higher organisms have evolved novel methods of coping with specific types of stress by utilizing novel chaperone system networks that can cope with the damage induced by the stress (Csermely, 2008; Palotai, Szalay, and Csermely, 2008). Furthermore, because multi-cellular organisms are composed of different tissue and organ systems, each cell type has adopted specific functions to deal with the specific stresses (i.e. liver-detoxification of exogenous compounds, kidneys and intestines-maintenance of osmotic balance, skin-protects against UV exposure and external chemical agents etc). Thus, the total 'stress' burden is distributed among many tissues, and not solely placed on any particular cell. Overall, because cancer is a general stress inducing disorder, it appears that HSF1 regulates the basal level expression of molecular chaperones that allow transformed cancer cells to cope with stress.

### **1.7. Thermotolerance: Protection from Cellular Damage by Molecular Chaperones**

The best examples of the cytoprotective actions of molecular chaperone come from thermotolerance studies (Lindquist, and Craig, 1988b;

Parsell, and Lindquist, 1993). In these studies, an organism is rapidly killed by heat exposure when it is switched from its normal growth conditions to extreme temperatures. However, organisms that have had been given mild preheat treatments, and thus contain higher amounts of Hsp's, survive the lethal heat exposure at much higher rates. Remarkably, heat pre-conditioning also allows the host to tolerate other forms of stresses, such as exposure to heavy metals, reactive-oxygen species (ROS), or hypoxia. With the exception of ionizing radiation, all forms of stresses results in increased levels of molecular chaperones (Anderson, Herman, *et al*, 1988), a fact that provides strong support for the hypothesis that molecular chaperones induce cytoprotection in response to a wide variety of stress conditions (Morimoto, 1998; Li, and Werb, 1982).

In yeast, the major proteins associated with thermotolerance acquisition include members of the Hsp100 family. Not surprisingly, exposure of preconditioned *Hsp104*<sup>-/-</sup> cells to a lethal heat shock (~50°C) results in the formation of large protein aggregates and a thousand-fold increase in cell death compared to wild-type cells under the same conditions (Sanchez, and Lindquist, 1990; Sanchez, Parsell, *et al*, 1993). Interestingly, antibodies targeted to yeast Hsp104 protein also recognize heat inducible proteins in *E. coli* and mammals (Parsell, Sanchez, *et al*, 1991). In mammals, *Hsp110*<sup>-/-</sup> knockouts have not yet been generated; however, overexpression of Hsp110, the mammalian Hsp104 homolog, results in an increased capacity to survive lethal heat shock exposures (Oh, Chen, and Subject, 1997).

In mammals, Hsp70 protein has been shown to be critical for thermotolerance acquisition (Lee, Kwon, *et al*, 2004). In this regard, adult *Hsp70*<sup>-/-</sup> animals show extreme heat sensitivity and display a dramatic

tendency to undergo apoptosis, a fact that will be discussed further in section 1.8 below. Similarly in *Drosophila*, Hsp70 is essential for thermotolerance acquisition, as animals deleted of all six copies of *Hsp70* genes do not acquire thermotolerance upon repeated stress exposure and cannot survive extreme heat shock exposures (Gong, and Golic, 2006; Sanchez, and Lindquist, 1990; Welte, Tetrault, *et al*, 1993). Currently many of the molecular events regarding thermotolerance acquisition are still not fully understood. It is tempting to speculate that thermotolerance acquisition depends on the concerted actions of Hsp110 and Hsp70, given that Hsp70 over-expression stabilizes Hsp104 (Sanchez, Parsell, *et al*, 1993), and that both proteins have been found to work together in the protein refolding pathway (Oh, Chen, and Subjeck, 1997).

### **1.8. Heat Shock Proteins are Molecular Chaperones that Catalyze Protein Folding Reactions**

Much of the current biochemical understanding of the activities and intermolecular interactions of molecular chaperones arise from *in vitro* experiments in which heat denatured polypeptides are incubated with various chaperones, and the activities, or structural status of the 're-natured' polypeptides are subsequently analyzed (Buchner, Schmidt, *et al*, 1991; Jakob, Gaestel, *et al*, 1993; Skowyra, Georgopoulos, and Zylicz, 1990; Wiech, Buchner, *et al*, 1992). In general, these studies demonstrated that the heat shock proteins Hsp27, Hsp60, Hsc70, Hsp90 and Hsp110 are capable of binding heat-denatured polypeptides and maintaining them in a 'folding-competent' state. However, complete protein renaturation does not occur until Hsp70 is recruited to the complex (Freeman, and Morimoto, 1996).

### **1.8.1. The Hsp70 family:**

During heat stress conditions, nearly all organisms over-express Hsp70 proteins. Strikingly, Hsp70 shows greater than 50% conservation among most living systems, primarily within the ATPase domain, and the peptide recognition domain (Lindquist, and Craig, 1988a; Hendrick, and Hartl, 1993). Our current understanding of the biochemical activities of Hsp70 stems from *in vitro* studies performed using purified DnaK protein isolated from *E. coli* (Bardwell, and Craig, 1984; Friedman, Olson, *et al*, 1984). DnaK, like Hsp70 proteins in higher organisms, is heat inducible and catalyzes protein-folding reactions in an ATP dependent manner by binding to exposed hydrophobic residues (Pelham, 1986; Rudiger; Skowyra, Georgopoulos, and Zylicz, 1990; Schumacher, Hurst, *et al*, 1994; Schumacher, Hansen, *et al*, 1996; Freeman, and Morimoto, 1996; Buchberger, and Bukau, 1997).

Interestingly, members of the *Hsp70* gene family have undergone several gene duplication events. In *S. cerevisiae*, at least nine genes related to the *Hsp70* family have been identified whose expression levels change in response to growing temperatures. In particular, the two main heat inducible Hsp70 isoforms are encoded by the *SSA3* and *SSA4* genes: both are expressed at low levels during normal growth conditions (23°C), and are dramatically up-regulated after heat stress (39°C) (Werner-Washburne, Stone, and Craig, 1987). *Drosophila* contains approximately 5-6 copies of *Hsp70*, as well as 7 other related genes that are abundantly expressed under normal growth conditions called heat shock cognate proteins (HSC's) (Wadsworth, 1982). Mammals express five *Hsp70* family proteins: Hsp70, Hsp72, p72, Grp78 and a mitochondrial specific Hsp70 isoform. The Hsp70 protein is

moderately expressed under normal growth conditions, is induced mildly by heat shock, and is commonly referred to in the literature as the '72k-heat shock protein' (Milarski, and Morimoto, 1986; Welch, and Feramisco, 1984). Hsp72 is strictly heat shock induced, and is referred to as Hsp70 (*Hsp70A1A*). The p72 protein is expressed at high levels in growing cells, while Grp78 is expressed at high levels under normal growth conditions and is commonly referred to as Bip (Watowich, and Morimoto, 1988). In the mitochondria, mHsp70 is expressed at high levels under normal growth conditions and is not heat inducible. Given the high degree of evolutionary conservation among Hsp70 proteins, it is not surprising that these HSP70 family members perform similar reactions within distinct cellular compartments: the endoplasmic reticulum lumen for GRP78, the cytoplasmic and nuclear compartments for p72, Hsp70, and Hsp72, and the mitochondria specific mHsp70. In yeast, any of the Hsp70 members (*SSA1-4*) can partially complement one another, thus further demonstrating the high degree of functional conservation among this family of proteins (Werner-Washburne, Stone, and Craig, 1987). To date, it is unknown whether the various mammalian Hsp70 isoforms can compensate for the loss of each other.

### **1.8.2. Refolding of heat denatured polypeptides by molecular chaperones**

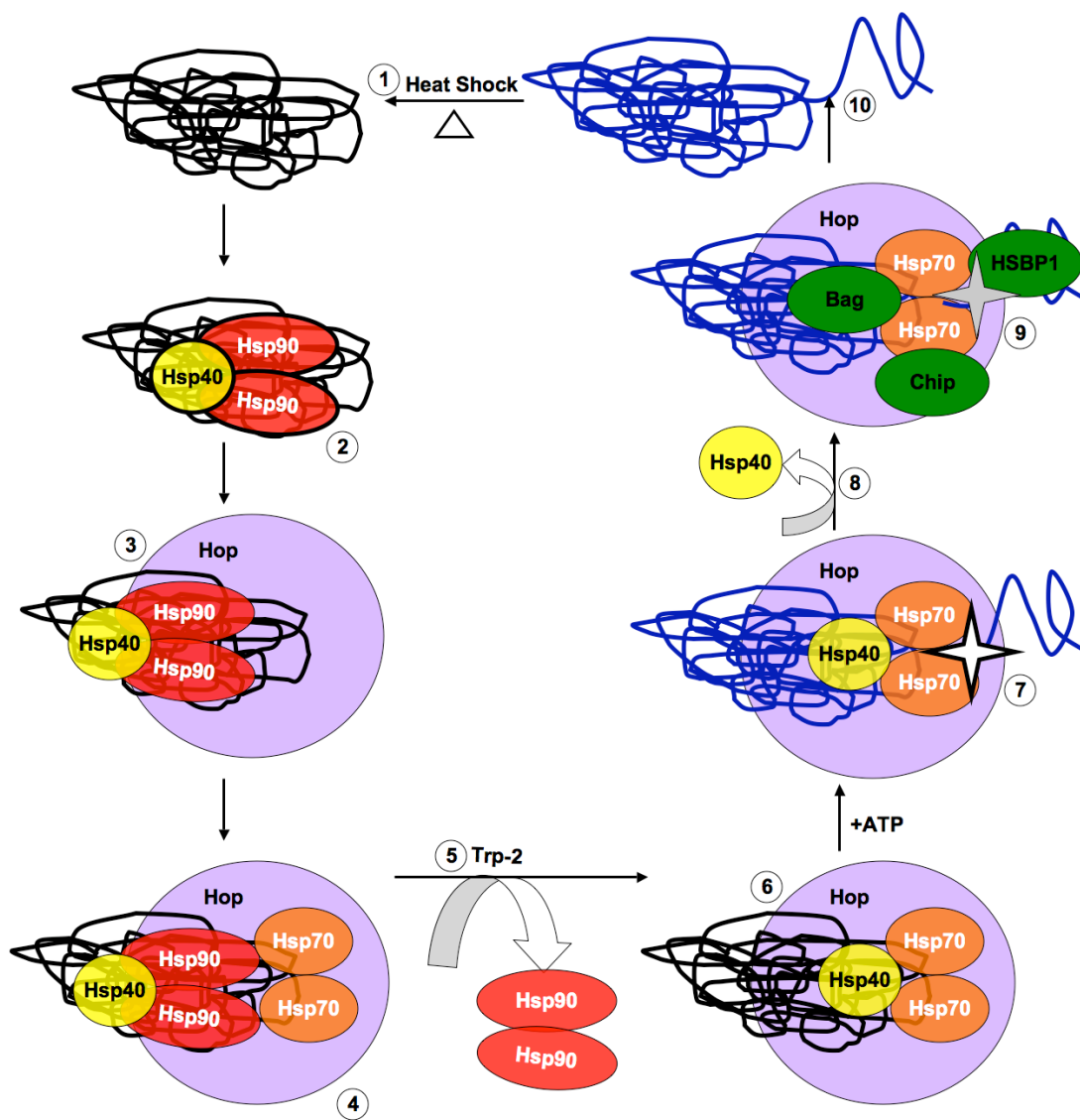
Protein refolding is an energy consuming reaction catalyzed by molecular chaperones. First, the unfolded protein substrate is delivered to the Hsp70 folding machinery by Hsp90/Hsp40 multi-chaperone complexes (Figure 1.4) (Michels, Kanon, *et al*, 1997; Takayama, Bimston, *et al*, 1997; Wiech, Buchner, *et al*, 1992). Binding of Hsp90/Hsp40 holds the unfolded protein

substrate in a conformation-competent state that awaits the arrival of Hsp70 (Schneider, Sepp-Lorenzino, *et al*, 1996; Wiech, Buchner, *et al*, 1992). In this regard, the heat denatured polypeptide/Hsp90/Hsp40 complex is brought to Hsp70 through the assembly of the scaffolding protein, Hop (Hsc70/Hsp90 organizing protein) (Pratt, and Dittmar, 1998) that recognize Hsp70 in an ADP bound form (Johnson, Schumacher, *et al*, 1998). Interestingly, Hop does not exhibit any chaperoning activity on its own, but rather induces protein renaturation by effectively bridging the interactions between the heat denatured polypeptide/Hsp90/Hsp40 complex and Hsp70 (Johnson, Schumacher, *et al*, 1998; Prapapanich, Chen, and Smith, 1998). Once Hsp90 is within close proximity to Hsp70, the denatured substrate is passed to Hsp70 by Tpr-2 co-chaperone (Brychzy, Rein, *et al*, 2003) and Hsp70 catalyzes the refolding process by hydrolyzing ATP. Once the protein substrate has been completely folded, it is released from the chaperone complex. First, Hsp40 is released from the multi-protein complex, followed by the recruitment of Bag1, Chip and HSBP1 (Zylicz, LeBowitz, *et al*, 1983; Chappell, Welch, *et al*, 1986; Buchberger, Valencia, *et al*, 1994; Buchberger, Theyssen, *et al*, 1995). These latter factors stabilize Hsp70 in an inactive, ADP bound form, resulting in the efficient release of the polypeptide from the Hsp70 chaperone complex. Interestingly, binding of Bag1/Chip/HSBP1 to the unfolded substrate or substrates affects the 'fate' of the polypeptide since those proteins that cannot be properly folded due to extreme damage are quickly targeted for degradation via the ubiquitin dependent proteosome pathway (Demand, Alberti, *et al*, 2001). Intriguingly, besides its role in protein re-folding, strong evidence suggests that Hsp70 is critical for the transport of proteins across lipid



**Figure 1.4: Protein Folding Pathway Mediated by Molecular Chaperones.**

Upon exposure to elevated temperatures, properly folded proteins (blue lines) become denatured (black lines)(1), and this results in the exposure of hydrophobic residues that are otherwise buried deep within the protein core. These heat-denatured polypeptides are quickly bound by molecular chaperones that maintain the proteins in a folding competent state (2) (i.e. Hsp90 & Hsp40). Binding of a scaffolding protein, Hop (3) to the Hsp90/40 chaperone complex promotes the recruitment of the folding apparatus, Hsp70 (4). Tpr-2 binding (5) to the multi-chaperone complex induces the release of Hsp90, and the passage of the heat denatured protein to Hsp70 (6). Once the polypeptide enters Hsp70 catalytic core, Hsp70 induces protein renaturation through the hydrolysis of ATP (7). After Hsp70 finishes refolding the polypeptide, and Hsp40 is released from the chaperone complex (8), followed by the subsequent recruitment of Bag, Chip and HSPB1 (9). Bag, Chip and HSPB1 maintain Hsp70 in a catalytically inactive (ADP) bound form. Depending on the status of the protein, Bag/Chip/HSPB1 determine the fate of the 'renatured' polypeptide. If the damage is too severe, and the protein cannot be properly folded, it is targeted to degradation via the ubiquitin dependent proteosome pathway. However, if the refolding reaction is successful (blue lines), the polypeptide is released from the chaperone complex (10).



membranes. Here, Hsp70 promotes protein-unfolding reactions before polypeptides can be transported across specific organelles (Deshaies, Koch, *et al*, 1988). In *S. cerevisiae*, the Grp78 homolog Kar2p temperature sensitive mutant contains high levels of partially translocated proteins caught in the act of passage through lipid membranes at the non-permissive temperature (Nguyen, Law, and Williams, 1991; Sanders, Whitfield, *et al*, 1992).

Recently, it has been found that the Hsp27 small heat shock protein family also facilitates the protein refolding processes by breaking apart pre-existing protein aggregates that accumulate during cellular stress (Kampinga, Brunsting, *et al*, 1994). Hsp27 maintains these “broken” polypeptides in a conformation-competent state (Jakob, Gaestel, *et al*, 1993), and protein renaturation occurs when Hsp70 is recruited to the complex (Ehrnsperger, Graber, *et al*, 1997). Similarly, the Hsp110 large heat shock protein family is known to promote refolding reactions by binding to heat denatured polypeptides and maintaining them in a conformation-competent state that requires subsequent Hsp70 activity (Oh, Chen, and Subjeck, 1997).

### **1.9. Regulation of Pro-survival Signaling Cascades by Hsp90/Hsp70 Chaperone Complexes**

Cancer is a disease of genetic instability, and while only a few mutations have shown to induce tumor formation, mature tumors have been shown to contain over 10,000 mutations at the time of diagnosis (Stoler, Chen, *et al*, 1999; Hahn, and Weinberg, 2002). Due to this high mutation rate, cancer cells have the ability to evade therapeutic treatment through the selection of drug resistant clones that are often associated with cancer relapse (La Rosee, Corbin, *et al*, 2002). Weinberg and colleagues suggest that during

cellular transformation, cells generally acquire six general characteristics: (1) self-sufficiency in growth signaling, (2) insensitivity to anti-growth signaling, (3) the ability to evade apoptosis, (4) sustained angiogenesis, (5) the ability to invade surrounding tissues and undergo metastasis, and (6) limitless replication potential (Hanahan, and Weinberg, 2000). Because of these general features, cancer cells have the capacity to survive extreme environmental conditions and therefore can colonize adverse microenvironments. Given the general adaptability to stress conditions, it is not surprising that cancer cells have an increased requirement for chaperone activity relative to non-cancerous cells, and indeed cancer cells have elevated amounts of heat shock proteins (Becker, Multhoff, *et al*, 2004). Previous attempts at isolating inhibitors targeting various molecular chaperones has resulted in failure, and has succeeded only for the Hsp90 family, geldanamycin and its derivatives (17-AAG and DMAG) (Whitesell, Mimnaugh, *et al*, 1994). In spite of this, it is now accepted that Hsp90 regulates the stability of proteins that promote each of the classical hallmarks of cancers: Her2, CDK's, Akt, Src, VEGF, Erb, and telomerase (An, Schulte, and Neckers, 2000; Bagatell, and Whitesell, 2004; Chiosis, Vilenchik, *et al*, 2004; Goetz, Toft, *et al*, 2003; Workman, 2004; Zhang, and Burrows, 2004). In most cases, Hsp90 inhibition induces the degradation of its client proteins, thus strongly suggesting that many of the mutated proteins that often promote cellular transformation are stabilized by the general activities of molecular chaperones (An, Schulte, and Neckers, 2000; Neckers, 2002; Mimnaugh, Xu, *et al*, 2004).

In the clinical setting, analyzing the levels of Hsp90 has become a good prognostic indicator of cellular transformation and metastatic potential of cancerous cells (Eustace, Sakurai, *et al*, 2004). Furthermore, Hsp90 inhibition

has been shown to greatly improve radiation sensitization (Bisht, Bradbury, *et al*, 2003), and increase the effective treatment of anaplastic cell lymphomas (Bonvini, Gastaldi, *et al*, 2002), acute myeloid leukemia (Minami, Kiyoi, *et al*, 2002), chronic myelogenous leukemia (Gorre, Ellwood-Yen, *et al*, 2002), prostate cancer (Vanaja, Mitchell, *et al*, 2002), as well as decrease metastasis in brain glioblastoma cells (Zagzag, Nomura, *et al*, 2003). Unfortunately, pharmacological Hsp90 inhibition also activates osteoclast c-Src signaling and promotes the invasion of prostate carcinoma cells in bone tissues (Yano, Tsutsumi, *et al*, 2008). Also, Hsp90 inhibition is known to induce cytoprotection by increasing expression levels of the anti-apoptotic factor Hsp70 (Bagatell, Paine-Murrieta, *et al*, 2000; Gabai, Budagova, and Sherman, 2005). In spite of these adverse effects, the Hsp90 inhibitors 17AAG and DMAG have reached phase II clinical trials, and are currently used in combination with other anti-cancer drugs during therapy. In this way, the *in vivo* efficacy of conventional chemotherapeutic drugs show dramatic improvements, resulting in an overall increase in the number of successfully treated cancer patients. Overall, it is becoming more evident that molecular chaperones maintain proteins that promote cellular transformation and tumorigenesis in humans.

#### **1.9.1 Chaperone maintenance of ligand-activated trans-membrane receptors which promote cellular transformation:**

The first insights that molecular chaperones affected ligand-activated hormone receptors came from findings that revealed that Hsp90 multi-chaperone complexes regulate the stability, ligand responsiveness, trafficking, and turnover rate of glucocorticoid receptors (GRs) (Catelli, Binart, *et al*, 1985;

Sanchez, Toft, *et al*, 1985; Schuh, Yonemoto, *et al*, 1985; Picard, Khursheed, *et al*, 1990). Together, Hsp90/70 maintain GRs in a ligand 'open' state to become highly sensitive to fluctuating glucocorticoid hormone levels (Bresnick, Dalman, *et al*, 1989; Hutchison, Dittmar, and Pratt, 1994; Nair, Toran, *et al*, 1996; Pratt, and Dittmar, 1998). Upon hormone binding, GR undergoes structural changes that result in GR binding to DNA and the subsequent induced expression of glucocorticoid responsive genes (Elbi, Walker, *et al*, 2004; Pratt, Galigniana, *et al*, 2004). At the plasma membrane, Hsp90/Hsp70 co-chaperone complexes also prevent GR from becoming targeted for degradation (Whitesell, and Cook, 1996; Bercovich, Stancovski, *et al*, 1997). This type of chaperone-mediated cell signaling control extends beyond the realm of GR receptors and includes other trans-membrane receptors, growth factors receptors, and non-receptor tyrosine kinases (Bardelli, Longati, *et al*, 1996). In many forms of cancers, the VEGF and ErbB-2 receptors exist in an abnormal conformation that require Hsp90 and Hsp70 activity (Le Boeuf, Houle, and Huot, 2004; Citri, Gan, *et al*, 2004; Kim, Chao, *et al*, 2004; Xu, Mimnaugh, *et al*, 2001).

### **1.9.2. Chaperone maintenance of non-receptor tyrosine kinases:**

The first protein kinase found to be regulated by molecular chaperones was the viral oncogene v-Src (Brugge, Erikson, and Erikson, 1981; Oppermann, Levinson, *et al*, 1981). Upon viral infection, v-Src binds Hsp90 and uses its chaperoning activity to promote the folding, stability, and assembly of viral proteins. Intriguingly, these studies found that Hsp90 is one of the most abundant molecular chaperones present under normal growth conditions, comprising ~1-2% the total protein content (Brugge, Erikson, and

Erikson, 1981; Oppermann, Levinson, *et al*, 1981). Since then, Hsp90 has been found to bind, and therefore regulate, hundreds of other signaling proteins (Pratt, and Toft, 2003), including but not limited to Raf, CK2, PKR, Ste11, CDK4 and IKK (Miyata, and Yahara, 1992; Cutforth, and Rubin, 1994; Van der Straten, Rommel, *et al*, 1997; Powers, Clarke, and Workman, 2008). Many of these Hsp90 client proteins have diverse functions and have been shown to be critical for viral infection, maintenance of cell proliferation, and apoptosis inhibition (Miyata, and Yahara, 1992).

The Raf oncogene, a component of the mitogen activated signaling or MAPK pathway, is a classic Hsp90 client protein. Using a genetic approach, Cutforth and colleagues found that Hsp90 modulates the MAPK signaling pathway in *Drosophila* (Cutforth, and Rubin, 1994; Van der Straten, Rommel, *et al*, 1997), and it was soon discovered that Hsp90 controls Raf mediated signaling activity in mammals as well (Grammatikakis, Lin, *et al*, 1999). Not relevant to protein kinases, but often found in a 'misfolded' conformation in many cancers, is the tumor suppressor protein p53. Interestingly, maintenance of the misfolded p53 mutants also requires Hsp70/Hsp90 chaperoning activity (Blagosklonny, Toretsky, *et al*, 1996; Pinhasi-Kimhi, Michalovitz, *et al*, 1986).

Overall, these studies suggest that Hsp90 multi-chaperone complexes regulate the signaling activity of pro-survival pathways. Therefore, future studies should focus on generating novel classes of inhibitors that effectively disrupt specific Hsp90 co-chaperone interactions present in transformed cancer cells (Kamal, Thao, *et al*, 2003; Powers, Clarke, and Workman, 2008).

### **1.10. Molecular Chaperones Antagonize Apoptotic Signaling Cascades at Multiple Levels**

Apoptosis is a naturally occurring process that organisms use to destroy unwanted cells in developmental processes like removal of supernumerary cells in the *Drosophila* eye, formation of limb buds in mammals, and is the main elicitor of cell death by anti-cancer drugs in humans. Depending on the initiating apoptotic stimuli, the external-death receptor pathway (Fas or TNF- $\alpha$  ligand activation) (Nagata, 1997), or mitochondria dependent signaling pathway (Li, Nijhawan, *et al*, 1997), the outer mitochondria membrane becomes permeable resulting in the release of various proteins that normally reside within the intermembrane space such as cytochrome-c, AIF-apoptotic inducing factor, and Smac/Diablo-caspase activating factors. Once in the cytosol, cytochrome-c induces formation of the apoptosome complex by inducing Apaf-1 dimerization and the recruitment of procaspase-9 (Li, Nijhawan, *et al*, 1997). Subsequently, the apoptosome activates a series of pro-caspase proteins that ultimately result in caspase-3 activation. Caspase-3 induces the cleavage of endogenous cellular proteins that results in loss of the integrity of lipid membranes, chromosome fragmentation, and, ultimately, cell death.

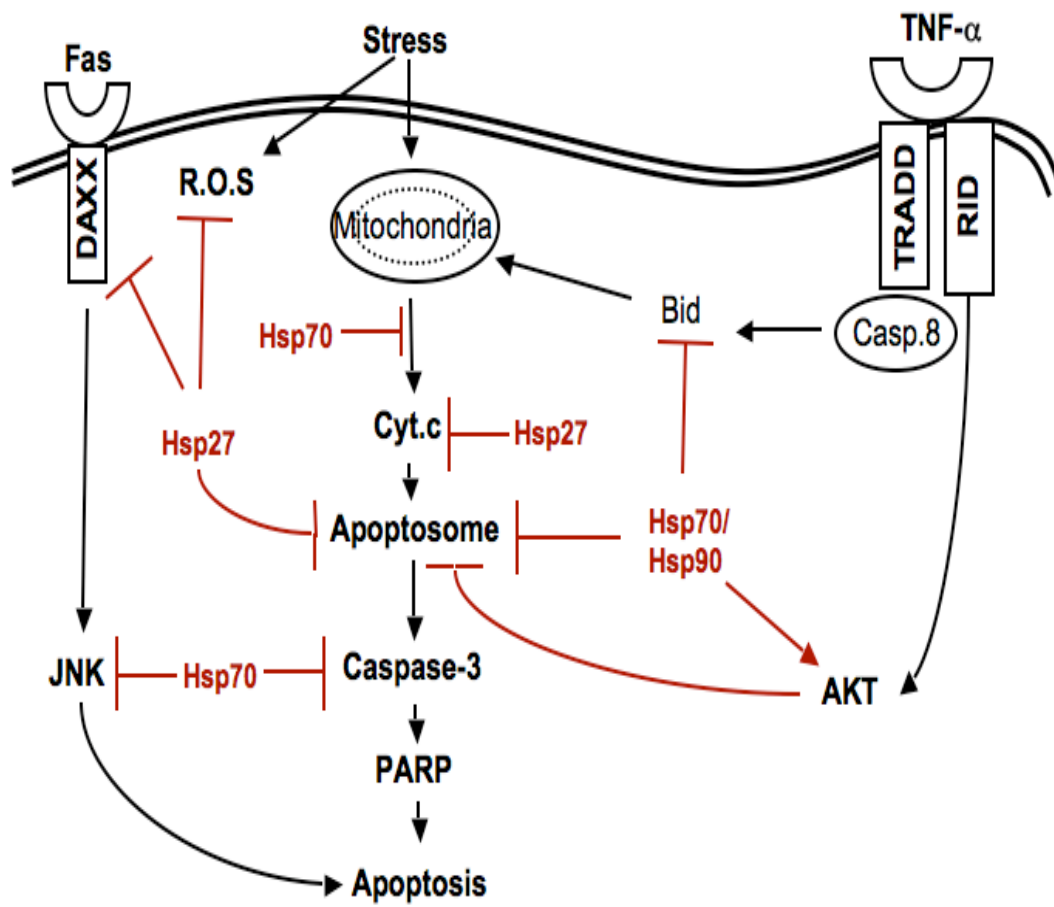
The regulation of cell survival by molecular chaperones has been the source of considerable debate; however, it is clear that molecular chaperones promote cell survival by attenuating apoptotic signaling at multiple levels (Figure 1.5). One protein in particular that has elicited some controversy regarding its function in apoptosis regulation is Hsp70. In this regard, it has been argued that the primary function of Hsp70 is to inhibit the apoptosome by interfering with Apaf-1 dimerization and procaspase-9 recruitment (Beere,



Wolf, *et al*, 2000). In contrast, Steel and colleagues argue that Hsp70 acts further upstream of the pathway by regulating cytochrome-c release from the mitochondria (Steel, Doherty, *et al*, 2004). Contrary to both of these reports, the Egeblad group argue that Hsp70's primary role does not involve cytochrome-c or apoptosome formation but rather the final signaling events, as well as maintaining procaspase-3 in an inactive form (Jaattela, Wissing, *et al*, 1998). Besides regulating the internal mitochondrial dependent apoptosis signaling pathway, Hsp70 has also been found to regulate the activity of death receptors TNF- $\alpha$  and Fas (Guo, Sigua, *et al*, 2005; Scaffidi, Schmitz, *et al*, 1999), which normally elicit apoptosis whenever a specific ligand binds to the extra-cellular ligand-binding domain (Figure 1.5). Once activated, death receptors recruit scaffolding proteins including Daxx, FADD, TRADD, and RIP, that are necessary for the recruitment and activation of procaspase-8. Procaspase-8 activation induces the release of cytochrome c from the mitochondria through Bid (Scaffidi, Schmitz, *et al*, 1999). Recently, it has been found that Hsp70 inhibits the ligand responsiveness, assembly of scaffolding proteins, and general signaling activity of death receptors in leukemia cells (Guo, Sigua, *et al*, 2005). Not surprisingly, *Hsp70*<sup>-/-</sup> derived cell lines undergo apoptosis at extremely high frequencies (Lee, Kwon, *et al*, 2004). Collectively, these studies indicate that the high Hsp70 levels observed among various cancers contribute to their survival by efficiently attenuating pro-apoptotic signaling, a classic hallmark of cancer cells.

The Hsp27 heat shock proteins, whose main function generally correlates with the disassembly of protein aggregates, and maintaining cytoskeleton integrity, has recently been shown to affect both the intrinsic and extrinsic apoptotic signaling pathways as well (Figure 1.5) (Charette, Lavoie,

**Figure 1.5: Regulation of apoptosis signaling pathways by molecular chaperones.** Molecular chaperones inhibit both stress induced intrinsic, or death receptor mediated extrinsic apoptotic signaling pathways at multiple levels. Black arrows: induction; Red hatch: inhibition. \*Note: Hsp90 stabilizes AKT, and this results in apoptosome inhibition (Apaf-9).



*et al*, 2000; Ehrnsperger, Graber, *et al*, 1997). In particular, Hsp27 inhibits apoptosis by the sequestering of cytochrome c from the mitochondria (Bruey, Ducasse, *et al*, 2000), the inhibition of apoptosome assembly (Garrido, Bruey, *et al*, 1999), and the Daxx death receptor scaffold proteins (Charette, Lavoie, *et al*, 2000), and the sequestering free-radicals and reactive oxygen species (ROS) (Mehlen, Kretz-Remy, *et al*, 1996). Interestingly, Hsp27 negatively regulates apoptosis as a large Hsp27 oligomeric complex (~1MDa) (Garrido, Bruey, *et al*, 1999; Bruey, Paul, *et al*, 2000). In mice, Hsp27 over-expression increases the tumorigenicity of rat colon cancer cells by effectively attenuating the death response (Garrido, Fromentin, *et al*, 1998). In human prostate cancer cells, Hsp27 RNAi depletion results in effective induction of apoptosis (Rocchi, So, *et al*, 2004), while injection of human breast cancer cells over-expressing Hsp27 into nude mice results in the formation of high-grade metastatic nodules (Lemieux, Oesterreich, *et al*, 1997).

Hsp90 displays intrinsic anti-apoptotic activity (Beere, Wolf, *et al*, 2000), as it can bind to and inhibit apoptosome complex formation (Apaf-1 dimerization), as well as inhibit procaspase-9 recruitment (Figure 1.5) (Pandey, Saleh, *et al*, 2000). However, relatively little is known about Hsp90's role in regulating components of the apoptotic signaling pathway. Instead, it is believed that Hsp90 exerts its general anti-apoptotic functions by maintaining proteins involved in cell survival signaling pathways (i.e. MAPK) that attenuate apoptotic-signaling through 'cross-talk' signaling interactions. In this regard, it's been shown that Hsp90 inhibition by 17-AAG & DMAG decreases AKT activity; AKT is known to maintain caspase-9 in an inactive state (Figure 1.5) (Basso, Solit, *et al*, 2002; Cardone, Roy, *et al*, 1998). Unlike *Hsp70*<sup>-/-</sup> derived cell lines, a true genetic analysis involving Hsp90's role in apoptosis has not

been performed since *Hsp90*<sup>-/-</sup> mice fail to survive beyond the 10<sup>th</sup> day of gestation (Voss, Thomas, and Gruss, 2000). Overall, the fact that cancer cells have increased levels of heat shock proteins, in particular Hsp70 and Hsp90 (Becker, Multhoff, *et al*, 2004; Nanbu, Konishi, *et al*, 1998), together with the fact that their inhibition greatly predisposes cancer cells to pharmacological treatment, emphasizes the importance of novel Hsp inhibitors aimed at developing more effective cancer therapies (Basso, Solit, *et al*, 2002; Bisht, Bradbury, *et al*, 2003; Jaattela, Wissing, *et al*, 1998; Nanbu, Konishi, *et al*, 1998; Powers, Clarke, and Workman, 2008; Soti, Nagy, *et al*, 2005; Wei, Zhao, *et al*, 1994; Zaarur, Gabai, *et al*, 2006).

#### **1.11. Modulation of the Heat Shock Response by Exogenous Compounds**

Having elevated amounts of heat shock proteins allow cells to better cope with the protein damage induced by various forms of stress, and over time, the beneficial functions of heat shock proteins has become apparent to humans. It has been suggested that societies that practice sauna as a method of socializing tend to retard the aberrant effects of aging, and societies that use sauna extensively are thought to 'age-better'. Intriguingly, the mild increases in temperature during the sauna process result in stress 'preconditioning', resulting in increased levels of molecular chaperones (Soti, and Csermely, 2003). Besides heat, a person's diet can dramatically affect the amount of heat shock proteins present in the system; for instance, consumption of large quantities of amino acids such as glutamine or metal ions such as Cu<sup>+2</sup>, Mn<sup>+2</sup>, and Zn<sup>+2</sup>, can also result in increased levels of heat shock proteins. In

mammals, zinc levels typically decrease during aging, and this event coincides with reduced levels of Hsc70, Hsp40 and Hsp60 proteins (Soti, and Csermely, 2003). Under these conditions, zinc depletion causes a dramatic increase in protein aggregates compared with same aged animals whose diet is supplemented with zinc. Similarly, caloric restriction is known to slow the aging process and elevate molecular chaperone levels. Diets that have increased levels of curcumin, a natural spice often found in Indian cuisine, increases endogenous Hsp70 levels (Khar, Ali, *et al*, 2001). Intriguingly, consumption of curcumin induces cytoprotection among normal cells yet induces apoptosis among a variety of cancer cell lines. However, studies show that curcumin treatment results in the clonal selection of drug resistant cancer cells that contain elevated heat shock proteins.

Small organic molecules can also modulate the heat shock response. In particular, the Hsp90 inhibitors geldanamycin and radicicol can induce heat shock gene expression when administered at low doses (Zou, Guo, *et al*, 1998; Koga, Xu, *et al*, 2006). Alternatively, as mentioned previously, the anti-inflammatory drug sodium salicylate effectively inhibits HSF1 trans-activation activity (Jurivich, Sistonen, *et al*, 1992; Westwood, and Wu, 1993). Recently, the naturally occurring compound, triptolide, has been found to attenuate the heat shock response. Because triptolide does not prevent HSF1 DNA binding, it is suggested that this drug inhibits the HSF1 activation domain or another component of the general transcriptional apparatus (Westerheide, Kawahara, *et al*, 2006). In any event, studies that utilize triptolide may reveal hidden properties of HSF1, or the transcriptional machinery, during the transcription cycle.

Other mechanisms that affect transcription regulation include a class of small non-coding RNA's (B2 RNA) transcribed by RNA polymerase III that are induced during heat shock conditions. In this mechanism, heat stress results in the expression of B2 RNA from interspread regions within the mouse genome thought previously to be 'junk DNA' (Allen, Von Kaenel, *et al*, 2004; Espinoza, Allen, *et al*, 2004). Expression of B2 RNA inhibits elongating competent RNA Pol II molecules at non-heat shock inducible genes without affecting heat-shock loci. Whether a similar gene regulatory mechanism exists among other eukaryotes is still unknown.

#### **1.12. Novel Uses of Aptamers in Basic and Clinical Applications**

Aptamers have emerged as promising reagents for dose-dependent modulation of protein activities both *in vitro* and *in vivo*. Aptamers are single-stranded DNA or RNA molecules that inhibit specific molecular surfaces by binding to their target via ionic, hydrophobic and hydrogen bond interactions. They are isolated through an iterative process of selection and amplification called Systematic Evolution of Ligands by EXponential enrichment (SELEX) (Ellington, and Szostak, 1990; Tuerk, and Gold, 1990) from combinatorial libraries containing approximately  $1 \times 10^{15}$  different molecules. The large sequence complexity associated with such a starting library ensures the best opportunity to isolate high affinity aptamers to various types of molecular surfaces, ranging from single small molecules (Sazani, Larralde, and Szostak, 2004) to distinct functional domains on a single protein (Shi, Fan, *et al*, 2007).

To generate RNA aptamers, the SELEX experiment starts with a large randomized sequence pool containing  $10^{14}$ - $10^{16}$  different species that fold into different shapes determined by their different sequences. This pool is then

subjected to iterative cycles of selection and amplification. In each cycle, a target, such as a protein molecule, is used to select from the pool of RNA molecules that bind it. Following the separation of the bound RNA from the unbound, the bound fraction is amplified by RT-PCR to generate a new pool for the next cycle. Usually RNA aptamers with the highest affinity for the target protein will dominate the population in 8-12 rounds. At the end of the process, the enriched aptamers are cloned and sequenced for further characterization. The aptamers generated by this process are capable of binding to a wide variety of targets with high affinity and specificity. To date, both DNA and RNA aptamers have been found to bind their targets with dissociation constants ( $K_d$ ) in the low nanomolar or picomolar range, and can discriminate between proteins that share common structural features (Fan, Shi, and Lis, 2005; Sevilimedu, Shi, and Lis, 2008).

In recent years, it has been demonstrated that RNA aptamers can be expressed *in vivo* under tight genetic control (Shi, Hoffman, and Lis, 1999), and that they can assert their effect within specific cells, tissues or developmental stages without eliciting an immune response in the targeted organism (White, Sullenger, and Rusconi, 2000). Our lab first pioneered the *in vivo* application of aptamers by targeting the *Drosophila* B52 protein. In this model system, we demonstrated that *Drosophila* B52 can be specifically inhibited *in vitro* and *in vivo* using a multivalent inhibitory aptamer RNA (iaRNA<sup>B52</sup>) (Shi, Hoffman, and Lis, 1999). This iaRNA<sup>B52</sup> binds B52 avidly, inhibits B52-stimulated pre-mRNA splicing *in vitro*, and suppresses all abnormal phenotypes caused by B52 over-expression *in vivo*. Since its initial demonstrated application, this method has been regarded as an effective therapeutic model and a novel approach to drug target validation (Famulok,



and Mayer, 1999; Pendergrast, Marsh, *et al*, 2005). In addition to their widespread utility as molecular probes in basic research (Fan, Shi, and Lis, 2005; Sevilimedu, Shi, and Lis, 2008), aptamers are quickly becoming used in the clinical setting (Blind, Kolanus, and Famulok, 1999; Bock, Griffin, *et al*, 1992; Csaky, 2003; Famulok, Blind, and Mayer, 2001; Lee, Canny, *et al*, 2005), thus demonstrating their broad utility as molecular inhibitors. Notably, an aptamer targeting NF- $\kappa$ B was recently expressed in cancer cell lines as well as a murine xenograft model and displayed significant anti-tumor activity (Mi, Zhang, *et al*, 2006). Therefore, to further understand HSF1 function during animal development and its role in tumor maintenance, I used the RNA aptamer technology in a chemical-genetic approach to inhibit HSF1 activity in both human cancer cells and *Drosophila*.

### **1.13. Concluding Remarks**

Eliciting an effective heat shock response allows cells to survive adverse conditions. Because of this, it is not surprising that this response became an integral component of living systems early during evolution. Intriguingly, rapidly evolving systems like cancer cells continue to exploit the benefits provided by molecular chaperones during processes such as cellular transformation and deregulated cancer growth. As the multiple functions of molecular chaperones become better understood, it is clear that in order to combat diseases like cancer, we must be able to effectively disrupt those molecular chaperones that maintain cancer cell survival. Only by systematically inhibiting the entire chaperone network will we be able to understand how each of these proteins contribute toward the general maintenance of the transformed phenotype associated with cancer. Using this

knowledge, we can begin to engineer novel inhibitors specific to the multiple chaperone-buffering systems present within cancer cells in order to ameliorate or irradiate this condition.

**CHAPTER TWO: EXPRESSION OF A NOVEL RNA APTAMER THAT  
DISRUPTS HSF1 ACTIVITY IN *DROSOPHILA* AND HUMAN CANCER  
CELLS.**

## 2.1 INTRODUCTION:

Heat shock factor (HSF) is a transcription factor that responds to a variety of signals to regulate the expression of a broad spectrum of target genes (Biggs, Zavitz, *et al*, 1994; Xiao, Zuo, *et al*, 1999). As mentioned previously in Chapter 1, *Drosophila* and *S. cerevisiae* each contain a single HSF1 gene, while in mammals and plants multiple isoforms exist that appear to have specialized functions (Czarnecka-Verner, Yuan, *et al*, 2000; Rabindran, Giorgi, *et al*, 1991; Sarge, Zimarino, *et al*, 1991; Schuetz, Gallo, *et al*, 1991) and is responsible for activating the heat shock response, a mechanism that is highly conserved among different kingdoms (Lindquist, and Craig, 1988a). During this response, HSF1 activates the expression of a specific set of heat shock genes, resulting in the accumulation of molecular chaperones that allow the organism to cope with cellular damage induced by the thermal stress. Among organisms that contain a single HSF1 gene, its activity is required at specific stages in the organism's life cycle (Wiederrecht, Seto, and Parker, 1988, Jedlicka, Mortin, and Wu, 1997; Walker, Thompson, *et al*, 2003; Morley, and Morimoto, 2004). However, higher organisms like mammals have evolved multiple HSF's that have specialized functions during animal development (Rabindran, Giorgi, *et al*, 1991; Sarge, Zimarino, *et al*, 1991; Schuetz, Gallo, *et al*, 1991; Xiao, Zuo, *et al*, 1999). Thus, HSF appears to have evolved multiple important regulatory roles in mammalian cells that extend beyond its role in the heat shock/stress response.

Although mammalian HSF1 activity is not required for animal viability, it does protect cells from various forms of stress (Murray, Whitfield, *et al*, 2004; Sarge, Zimarino, *et al*, 1991; Trinklein, Murray, *et al*, 2004) and also has been implicated in cancer as a "non-classical oncogene". HSF1 activity promotes

tumor formation and maintenance of the transformed cancer cell phenotype without affecting the viability of normal cells (Dai, Whitesell, *et al*, 2007). Therefore, HSF1 functions to promote cell survival even under conditions that could potentially become deleterious to the host, such as development of the transformed state.

To further understand HSF1 function during animal development and its role in tumor maintenance, I utilized the RNA aptamer technology in a chemical-genetic approach to inhibit HSF1 activity in both *Drosophila* and human cancer cells. In this chapter, I report the design, construction, and validation of a potent inhibitory aptamer RNA molecule for HSF1 called iaRNA<sup>HSF</sup>. This iaRNA<sup>HSF</sup> contains two HSF1 binding domains engineered from a previously isolated RNA aptamer that targets the highly conserved HSF1 DNA binding domain-Linker region (Zhao, Shi, *et al*, 2006). In *Drosophila*, I demonstrate that this iaRNA<sup>HSF</sup> can interfere with HSF1 trans-activation function under both non-induced and heat shock conditions *in vivo*. Under non-stressed conditions, systemic iaRNA<sup>HSF</sup> expression results in decreased HSF1 occupancy at the Hsp83 (63B) genomic locus, resulting in a significant decrease of transcribed *Hsp83* mRNAs. Additionally, *Drosophila* animals that express iaRNA<sup>HSF</sup> display morphological abnormalities that resemble known *Hsp83* mutants at high frequencies, consistent with previous reports that implicate Hsp83 in animal trait variation (Rutherford, and Lindquist, 1998; Sollars, Lu, *et al*, 2003; Yeyati, Bancewicz, *et al*, 2007; Yue, Karr, *et al*, 1999). I also demonstrate that during thermal stress iaRNA<sup>HSF</sup> expression compromises HSF1 activity, resulting in the attenuation of the heat shock response. I also confirm the functional specificity of iaRNA<sup>HSF</sup>-HSF1 interaction using a reciprocal suppression test in which the developmental

defects induced by iaRNA<sup>HSF</sup> or HSF1 over-expression are effectively attenuated by the co-expression of the constructs. Lastly, because of the broad implication of increased levels of heat shock proteins in diseases such as human cancer (Dai, Whitesell, *et al*, 2007; Kang, Plescia, *et al*, 2007; Soti, Nagy, *et al*, 2005; Xiao, Pacyna-Gengelbach, *et al*, 2005; Zaarur, Gabai, *et al*, 2006), I examined the effect of iaRNA<sup>HSF</sup> under conditions that model cellular transformation in flies and mammalian cancer cells. In *Drosophila*, HSF1 inhibition by iaRNA<sup>HSF</sup> suppresses the abnormal phenotypes that arise from expression of gain-of-function mutants of the Epidermal Growth Factor Receptor (EGFR) *ellipse* mutant, and Raf oncogenes within the wing and eye tissues, respectively.

The phenotypic effects of iaRNA<sup>HSF</sup> expression are similar to *Hsp83* mutants to, or treatment of EGFR<sup>*Ellipse*</sup> and Raf mutant flies with the Hsp83 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG), a frequently used anticancer agent in humans (Sawai, Chandarlapaty, *et al*, 2008). In human cancer cells, iaRNA<sup>HSF</sup> shows the properties of a potent anti-tumor drug with activities comparable to those of 17-AAG. In particular, iaRNA<sup>HSF</sup> induces a cancer-specific apoptotic response that correlates with the depletion of Hsp70 and Hsp90 family members, essential components of a larger chaperone network thought to be required for the maintenance of the transformed phenotype associated with cancer. Collectively, my results indicate that HSF1 inhibition by iaRNA<sup>HSF</sup> attenuates mitogenic growth signaling and inhibits transformed growth, thus making iaRNA<sup>HSF</sup> a promising new reagent worthy of further characterization in whole animal cancer studies.

## 2.2. RESULTS

### **2.2.1. Design, construction, and validation of the iaRNA<sup>HSF</sup> expression system**

Previously, our group isolated and characterized an RNA aptamer (AptHSF-RA1) that recognizes the DNA binding-linker domain of the *Drosophila* HSF with an apparent dissociation constant ( $K_d$ ) of 20-40nM (Zhao, Shi, *et al*, 2006). Shi H. *et al* previously developed an efficient scheme to deliver RNA aptamers as synthetic genes in transgenic flies to test their *in vivo* effects (Shi, Hoffman, and Lis, 1999). Here I have adapted this general delivery method to design and construct a genetic system for the expression of AptHSF-RA1.

In particular, I have taken a minimized version of the aptamer and augmented it in two ways. First, in order to effectively target the multi-domain HSF protein, I designed a divalent version by joining together two identical aptamers through two hinges and a three-way junction (Figure 2.1A). Analogous to the F(ab')<sub>2</sub> fragment of an antibody, this configuration dramatically increased the avidity of the resulting RNA molecule to HSF. Second, the molecule contains a self-cleaving hammerhead ribozyme that ligates the free ends of the cleaved RNA, resulting in increased RNA stability while at the same time making it possible to generate block polymers of the coding sequence so that a single synthetic polymeric template can code for multiple aptamer RNAs from every transcription cycle (Shi, Hoffman, and Lis, 1999). The resulting molecule, as shown in Figure 2.1A, is named inhibitory aptamer RNA HSF (iaRNA<sup>HSF</sup>).

To determine the apparent affinity of our engineered dimeric construct to HSF1, I performed electrophoretic mobility shift assays (EMSA) using

excess purified dHSF protein and limiting amounts of *in vitro*-transcribed,  $^{32}\text{P}$ -UTP-substituted iaRNA<sup>HSF</sup> (<1nM) (Figure 2.1B). The results demonstrate that the designed dimeric construct improves the apparent affinity of the original monomeric HSF aptamer several fold, yielding an apparent  $K_d$  of ~8nM (Figures 2.1 B&C). This improved avidity is sufficient to prevent HSF from binding to its natural promoter sequences, as seen from gel shift competition assays using limiting amounts of radiolabelled *Hsp70* and *Hsp83* promoter DNA (<1nM). In these experiments, the addition of increasing amounts of cold iaRNA<sup>HSF</sup> inhibits the formation of an HSF/DNA promoter complex relative to the addition of a control yeast tRNA molecule (Figure 2.2A). Under these conditions, inhibition of HSF binding to the *Hsp83* promoter requires approximately 5-fold more iaRNA<sup>HSF</sup> than does the inhibition of HSF binding to *Hsp70* promoter (Figure 2.2B). These differences are likely a consequence of the spacing and arrangement of the HSF binding sites between the *Hsp83* and *Hsp70* promoters (Fernandes, Xiao, and Lis, 1994; Fernandes, Xiao, and Lis, 1995).

To determine if iaRNA<sup>HSF</sup> inhibits HSF1 transcriptional activity in the complex milieu of a whole cell extract, I added increasing amounts of non-radiolabelled iaRNA<sup>HSF</sup> to yeast whole cell extracts expressing HSF1 and incubated the reaction in an *in vitro* G-less cassette transcription template that contains HSF1 binding sites, heat shock elements (HSE) within the promoter (Figure 2.3). In this system, one can directly measure the inhibitory potential of an aptamer in whole cell extracts because transcription of the reporter occurs when ATP, CTP and UTP is added to the reaction. To visualize the final transcript, a small amount of radiolabelled UTP was added.

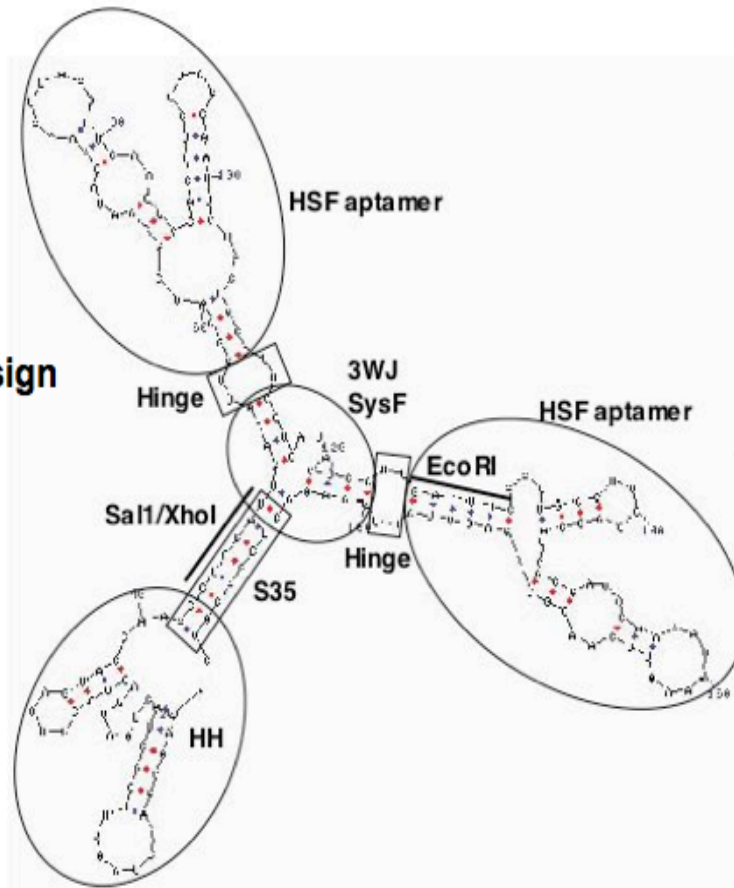


**Figure 2.1: iaRNA<sup>HSF</sup> binds to HSF avidly, K<sub>d</sub>=8nM.**

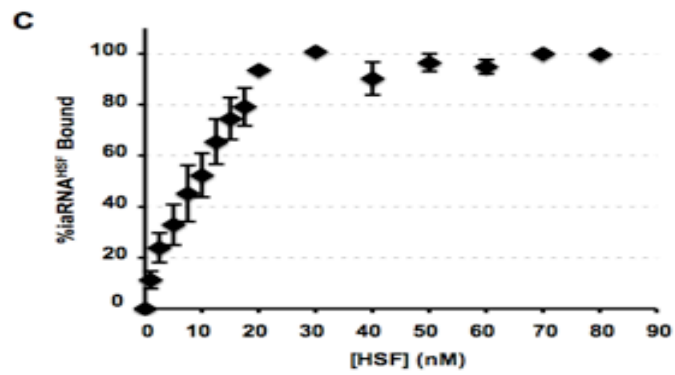
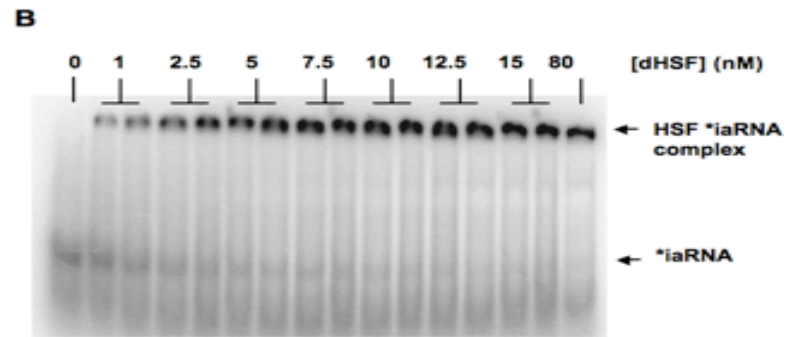
A). Lowest energy diagram predicting the secondary structure of the aptamer RNA using M-fold. iaRNA<sup>HSF</sup> DNA sequence (green letters correspond to the hammerhead ribozyme and red letters corresponds to the HSF aptamer sequence). B). Electrophoretic motility shift assay (EMSA) using radiolabelled iaRNA<sup>HSF</sup> (1nM) and increasing amounts of dHSF protein shows that the aptamer RNA binds to its target avidly. C). Quantification of independent EMSA assays reveals the apparent affinity of the iaRNA<sup>HSF</sup> K<sub>d</sub>=8 nM (n=5, error indicates %SEM) (Figure is displayed on multiple pages)

**A**

**iaRNA<sup>HSF</sup> Design**

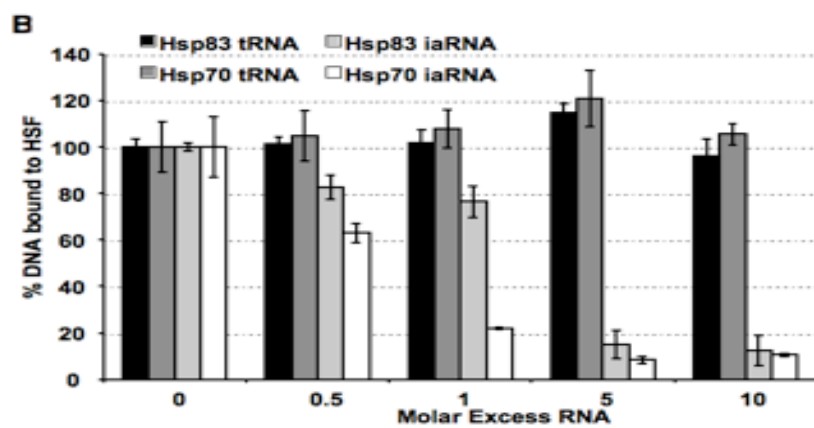
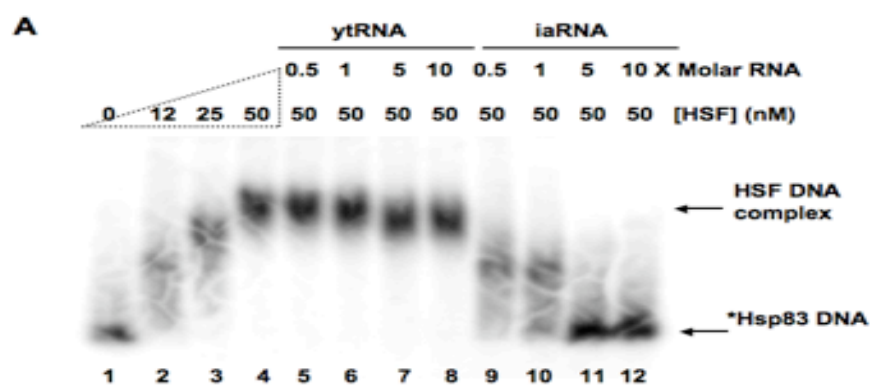


5' **CTCGAG**tgacggtt**GGCATCGCGATACAAAATTAAGTTGAACGCGAGT**tcttcgga**ATTCAACTGC**  
**C**ttcgtcatactccttg**AATTC**AACTGCC**t**tcg**GGCATCGCGATACAAAATTAAGTTGAACGC****GAGT**  
**T**cttgGagg**CTCGACGTCTAGCGATGTGGTTTCGCTACTGATGAGTCCGTGAGGACGAAACGTCGAC**



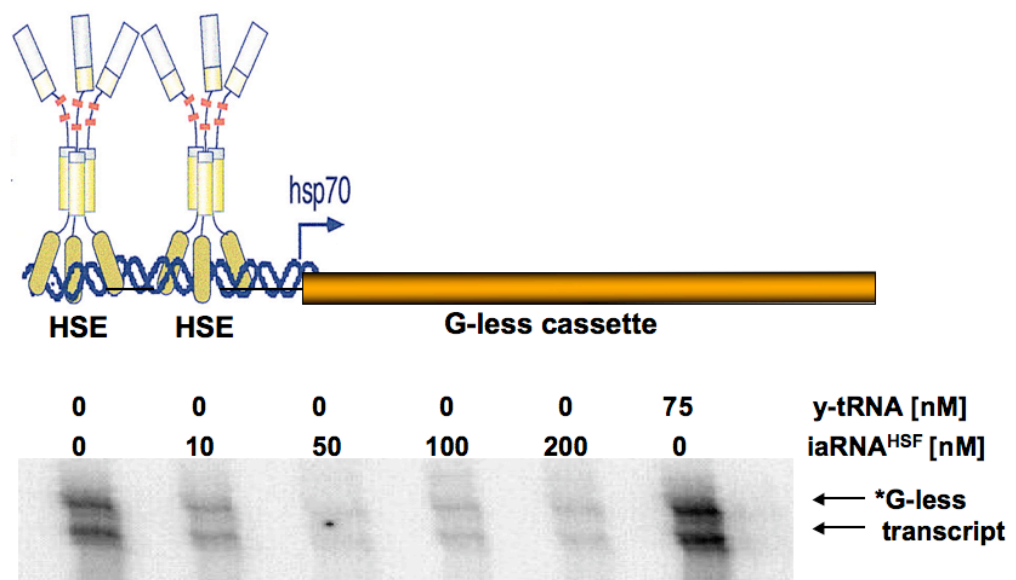
**Figure 2.2: iaRNA<sup>HSF</sup> competes with HSF DNA binding at native promoters *in vitro*.**

A). HSF EMSA using limiting amounts of Hsp83 promoter DNA (1nM)(-449>+114) (lanes 2-12), and increasing molar (M) concentrations of cold “non-radiolabelled” iaRNA<sup>HSF</sup> (lanes 9-12) or yeast tRNA (lanes 5-8). B). Quantification of competition experiments by filter binding assays using Hsp83 (-449>+114) or Hsp70 (-200>+64) promoter DNA. Data normalized to highest yeast tRNA signal (Hsp70 %SEM n=3, Hsp83 %SEM n=6).



**Figure 2.3: iaRNA<sup>HSF</sup> inhibits HSF1 induced transcription *in vitro*.**

Addition of non-radiolabelled iaRNA<sup>HSF</sup> to yeast whole cell extracts inhibits HSF1 ability to induce transcription from a G-less cassette that contains heat shock regulatory elements (HSE) upstream the start site. Effective transcription inhibition is first observed at ~10nM iaRNA<sup>HSF</sup>.

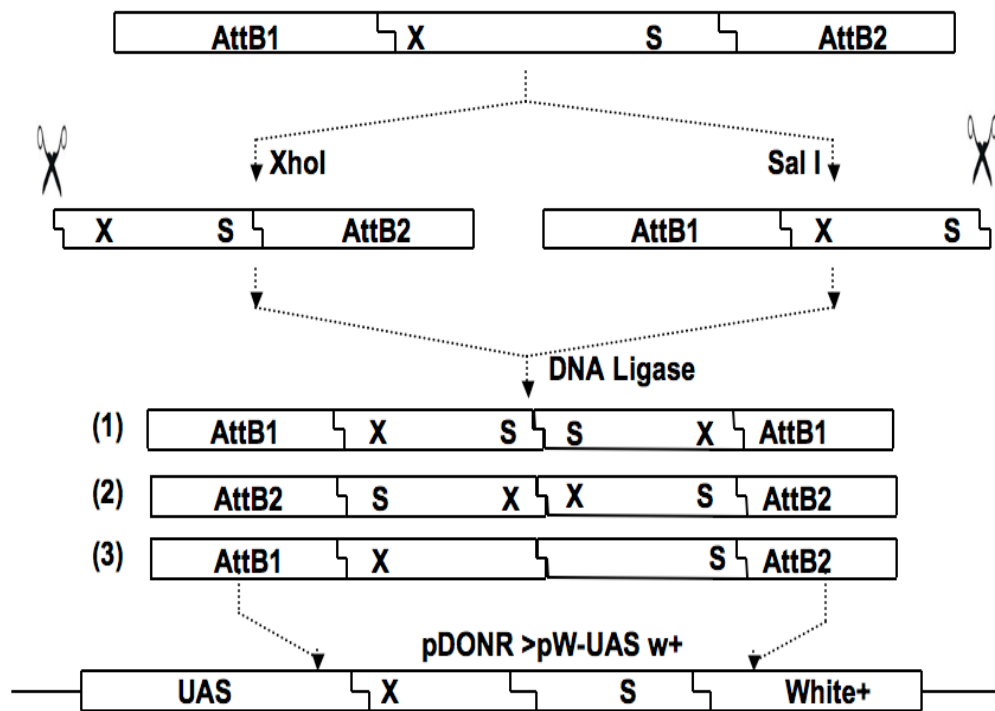


The results show that iaRNA<sup>HSF</sup> significantly compromises HSF1 transcriptional activity even at the lowest aptamer concentration (10nM).

In *Drosophila*, as in most eukaryotes, the amount of gene product generally depends on gene dosage; therefore, more copies of iaRNA coding sequence introduced into an animal or cell results in higher iaRNA levels. To increase iaRNA production and accumulation in animals, I developed a new cloning strategy that systematically generates repetitive head-to-tail iaRNA coding genes that double in size during every cloning cycle (Figure 2.4). This expression system allows for high-level expression of aptamer RNAs because each repeating aptamer gene within a given polymeric template is flanked by a self-cleaving hammerhead ribozyme (Figure 2.5A). Upon transcription of the polymeric template RNA, the hammerhead ribozyme undergoes self-cleavage, resulting in the release of multiple free functional iaRNA's from every transcription cycle (Shi, Hoffman, and Lis, 1999). This self-cleavage insures that the aptamer RNAs are not polyadenylated for export and remain localized within the nucleus. In addition, the hammerhead ribozyme undergoes a self-ligation reaction that produces covalently closed circles that protect RNA molecules from degradation (Shi, Hoffman, and Lis, 1999). To determine whether our engineered construct does in fact undergo self-cleavage when transcribed from a polymeric DNA template, I performed time dependent *in vitro* transcription reactions using radiolabelled <sup>32</sup>P-UTP as substrate. In these experiments, the hammerhead ribozyme undergoes self-cleavage as observed by the appearance and accumulation of the mature iaRNA<sup>HSF</sup> (200 basepairs) from a nascent transcript (487 bases) within the first few minutes of the reaction (Figure 2.5B). Because the fully processed form of the aptamer

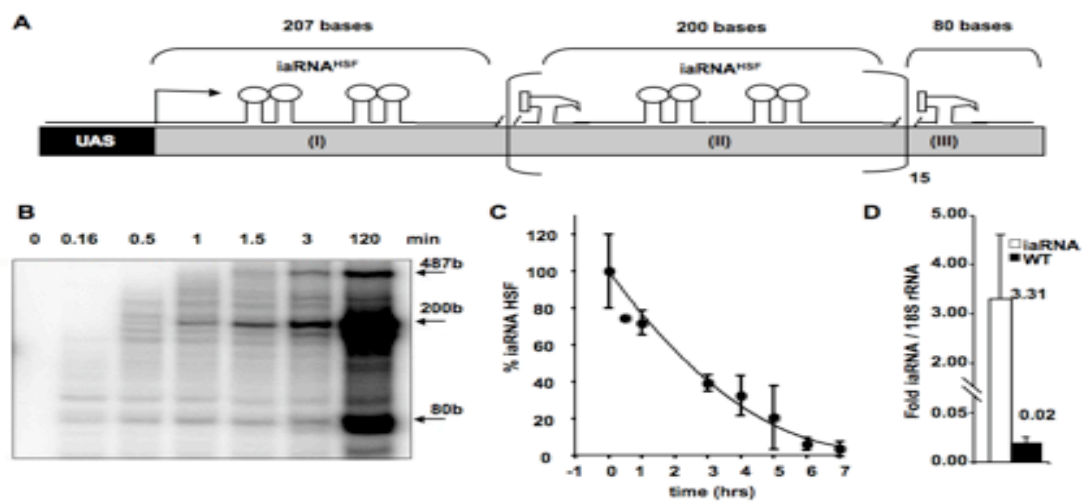


**Figure 2.4: Polymerization of iaRNA<sup>HSF</sup> genes results in the synthesis of multiple aptamers from each round of transcription.** Strategy for the polymerization of the iaRNA<sup>HSF</sup> gene: Gateway<sup>®</sup> cloning sequences, AttB<sub>1</sub> and AttB<sub>2</sub>, flank the iaRNA<sup>HSF</sup> gene that contains two HSF aptamers upstream of a hammerhead ribozyme, a Xho1 (5') site, and a Sal1 (3') site. Each Gateway<sup>®</sup> ready unit is digested with Xho1 or Sal1 and the resulting digests are ligated together, creating a polymer of 2 iaRNA<sup>HSF</sup> genes. Only fragment #3 results in the proper head to tail orientation of the polymeric gene and the appropriate Gateway<sup>®</sup> flanking sequences. Using the polymer of 2 as a template and repeating the dimerization strategy creates a polymer of 4. Overall, geometric progression of polymeric length is achieved in each subsequent round of dimerization.



**Figure 2.5: Design of iaRNA<sup>HSF</sup> expression system *in vivo*.**

A). Diagram of a polymeric template of 16 iaRNA<sup>HSF</sup> gene units and their corresponding transcripts (i) corresponds to the first processed iaRNA<sup>HSF</sup> which lacks a hammerhead, (ii) corresponds to subsequent iaRNA<sup>HSF</sup> that contain the “self-cleaving” hammer head ribozymes, and (iii) corresponds to the final processed hammer head ribozyme that does not bind to HSF). B). *In vitro* transcription of a polymer of 2 iaRNA<sup>HSF</sup> repeats demonstrates the effective release and accumulation of iaRNA<sup>HSF</sup> (200 bases), from its full length transcript (487 bases). Transcription reactions were performed using T7 Polymerase and radiolabelled <sup>32</sup>P-UTP. C). Determination of iaRNA<sup>HSF</sup> stability in *Drosophila* S2 cell shows that iaRNA<sup>HSF</sup> has an apparent half-life ( $t_{1/2}$ )~2hrs. iaRNA<sup>HSF</sup> levels are plotted as a function of time after inhibition of Pol II transcription with  $\alpha$ -amanitin (values normalized to 18S rRNA and quantified by RT-qPCR, n=3). D). Quantification of iaRNA<sup>HSF</sup> levels relative to 18S rRNA among animals that express iaRNA<sup>HSF</sup> (white bar) or parental controls that contain the iaRNA<sup>HSF</sup> genes but lack Gal4 protein (black) (%SEM n=3).



appears prior to the full-length transcript, self-cleavage occurs before the T7 polymerase can reach the end of the aptamer array.

To test the stability of iaRNA<sup>HSF</sup> in living cells, I generated stable *Drosophila* S2 cells that contain 8 tandem iaRNA<sup>HSF</sup> coding sequences downstream from the inducible metallothienin promoter. I induced the production of iaRNA<sup>HSF</sup> with 0.5mM copper sulfate for 24hrs, blocked further transcription with alpha-amanitin, and analyzed the rate of iaRNA<sup>HSF</sup> decay by quantifying the levels of iaRNA relative to 18S ribosomal RNA at specific times using reverse transcription quantitative PCR (RT-qPCR). These experiments show that the *in vivo* half-life of iaRNA<sup>HSF</sup> is 2 hours, which compared to the turn over rate of most endogenous RNAs (minutes), is a dramatic improvement in overall RNA stability (Figure 2.5C).

To generate *Drosophila* stocks that express iaRNA<sup>HSF</sup> in specific tissues or developmental stages, I created transgenic fly lines that contain 8 and 16 repeating iaRNA<sup>HSF</sup> units downstream from the inducible Gal4/UAS promoter (Brand, and Perrimon, 1993) using P-element transformation vectors. High level iaRNA<sup>HSF</sup> was achieved by recombining multiple aptamer genes on a single chromosome. To preserve this high-level iaRNA<sup>HSF</sup> construct, a *Drosophila* line expressing ~50 aptamer genes was produced from individual genomic insertions containing 8 and 16 repeating iaRNA<sup>HSF</sup> genes. By expressing Gal4 protein using the tubulin promoter in this genetic background, iaRNA<sup>HSF</sup> can be induced in whole animals to approximately 3 times the molar concentration of 18S rRNA, or ~150 fold higher than parental strains that lack Gal4 (Figure 2.5D). Therefore, this expression system is highly effective for achieving high iaRNA<sup>HSF</sup> expression in *Drosophila*.

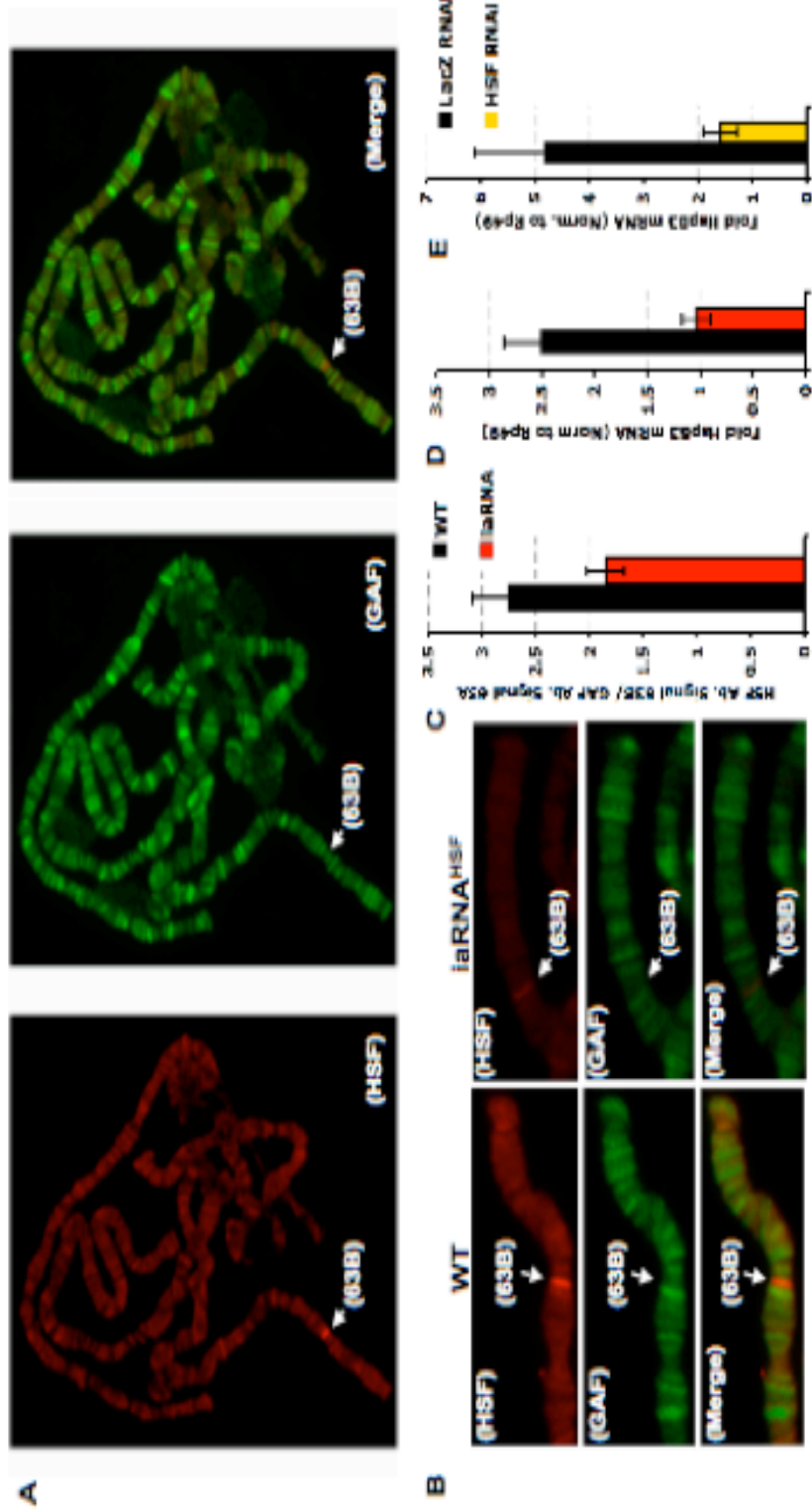
### **2.2.2. Efficacy of iaRNA<sup>HSF</sup> as a HSF antagonist**

To determine the *in vivo* efficacy of iaRNA<sup>HSF</sup> as a HSF1 antagonist, I measured its effect on known HSF gene targets under both non-heat shock and heat shock conditions. I focused my attention on the *Hsp83* gene (63B locus) based on the fact that under non-stress inducing conditions HSF is significantly enriched at this locus relative to any other chromosomal site (Westwood, Clos, and Wu, 1991; Yao, Munson, *et al*, 2006). I investigated the effects of iaRNA<sup>HSF</sup> expression at three different levels: HSF binding to the *Hsp83* chromosomal locus, inhibition of *Hsp83* mRNA levels, and whole *Drosophila* phenotypes. Immunofluorescent antibody staining for HSF on *Drosophila* salivary gland chromosomes confirms that under normal growth conditions HSF preferentially binds to the *Hsp83* locus (63B) (Figure 2.6A, compare HSF signal (red) relative to GAF control (green)). However, upon iaRNA<sup>HSF</sup> expression, the HSF levels at the 63B locus is effectively reduced by approximately 50% ( $p=0.035$ ) (Figure 2.6B-C).

Previous reports have implicated the enhancer elements and upstream regulatory sequences present in the promoter regions of the *Hsp83* gene as being critical for expression (Xiao, and Lis, 1989). Because no direct evidence has yet been reported to determine HSF's role in the constitutive expression of *Hsp83*, I decided to test whether HSF inhibition compromises *Hsp83* expression by quantifying the levels of *Hsp83* mRNA in iaRNA<sup>HSF</sup>-expressing and wild type (Gal4 parental) *Drosophila* animals. Here, animals that express iaRNA<sup>HSF</sup> have ~50% less *Hsp83* mRNAs compared to control animals ( $p=0.008$ ) (Figure 2.6D), thus indicating that under non-heat-shock conditions HSF activity is required for the expression of *Hsp83*. I confirmed this result using another approach that involved depleting HSF from

**Figure 2.6: Constitutive iaRNA<sup>HSF</sup> expression inhibits HSF activity at *Hsp83* (63B locus) under NHS conditions.**

A). HSF distribution within WT *Drosophila* chromosomes shows it is most abundant at *Hsp83* gene (63B locus) under non-induced conditions (NHS). Antibodies: Red=HSF, Green=GAF (control). B). Constitutive iaRNA<sup>HSF</sup> expression results in decreased HSF binding to *Hsp83* gene (63B locus) during NHS conditions. C). Quantification of the relative intensities shown in panel B in WT (n=29) and iaRNA<sup>HSF</sup> expressing animals (n=29). (Observed signals normalized to GAF intensities at 63A). D). Constitutive iaRNA<sup>HSF</sup> expression results in decreased *Hsp83* mRNAs under NHS conditions. (Quantification of *Hsp83* mRNAs in WT and iaRNA<sup>HSF</sup> expressing animals using RT-qPCR. WT n=6, iaRNA<sup>HSF</sup> n=6). E). HSF RNAi depletion within *Drosophila* S2 cells results in decreased *Hsp83* mRNAs similar to the iaRNA<sup>HSF</sup> effects observed in panel D (mRNAs quantified by RT-qPCR. Error %SEM, LacZ RNAi “WT” n=4, HSF RNAi n=4).





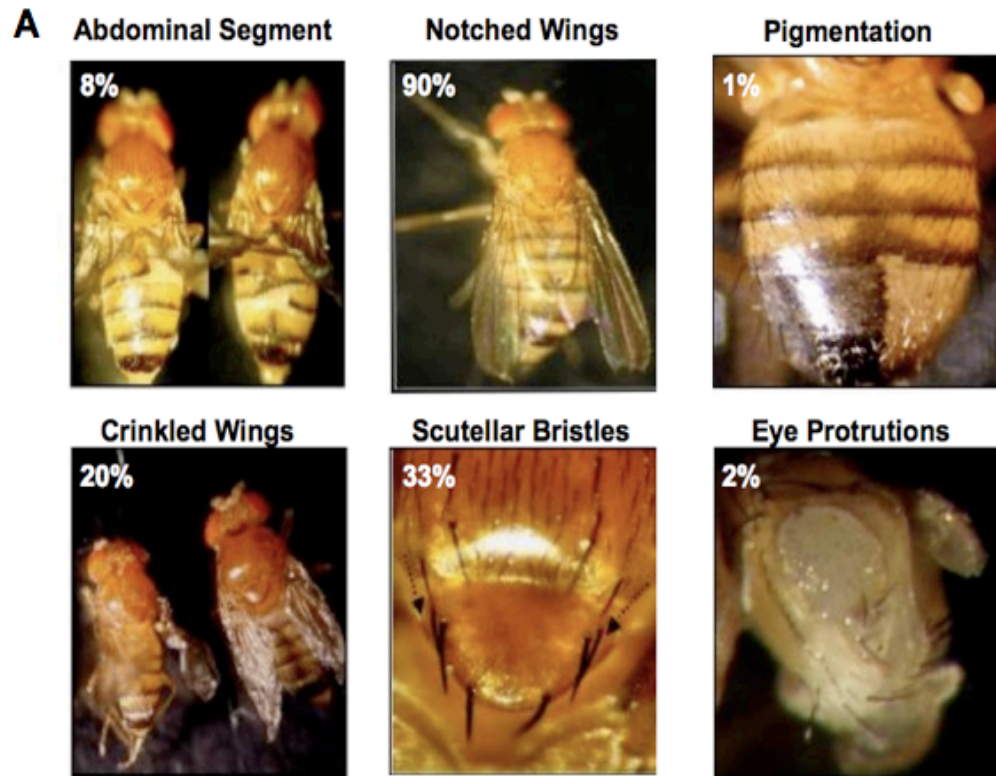
*Drosophila* S2 cells using RNAi. In these experiments, HSF knockdown resembled the effects of iaRNA<sup>HSF</sup> expression and reduced *Hsp83* transcript levels by 50% (p=0.017) (Figure 2.6E, compare HSF RNAi versus LacZ RNAi treatment), thus further demonstrating that normal HSF activity is required for the expression of *Hsp83* mRNA under normal growth conditions.

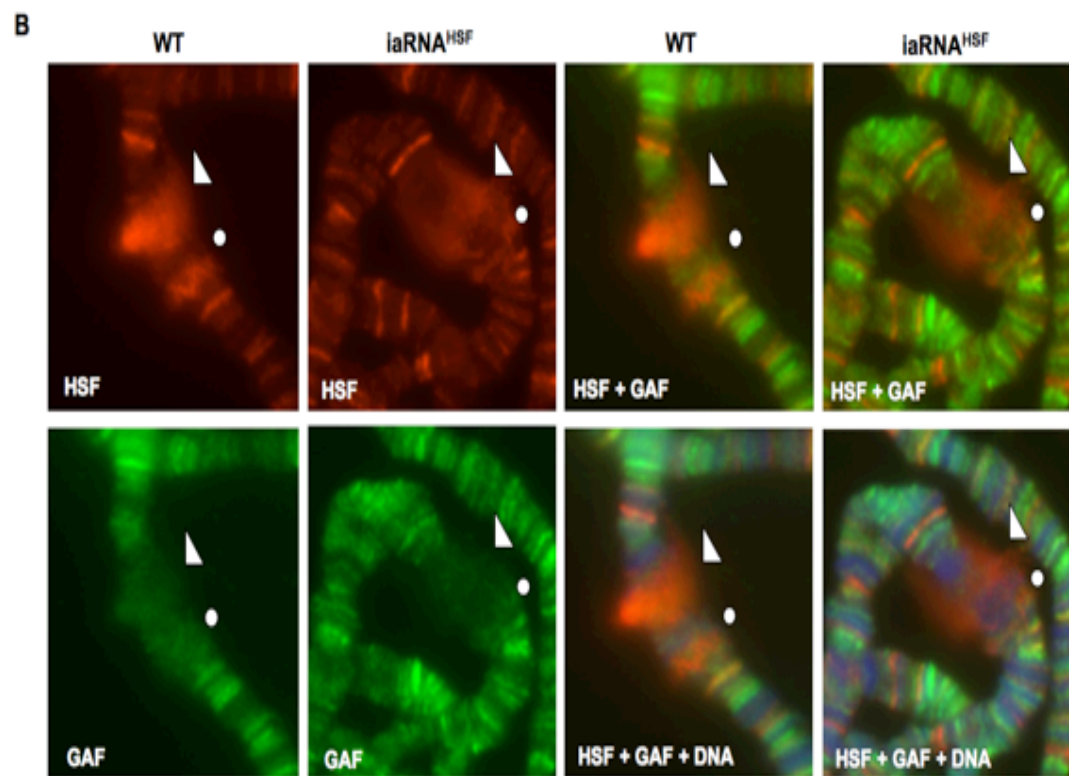
Adult *Drosophila* that constitutively express iaRNA<sup>HSF</sup> display abnormal abdominal segments, wing shape and morphology, bristles, and eye protrusions in specific genetic backgrounds at high frequencies (Figure 2.7A). Intriguingly, these aptamer-induced phenotypes closely resemble the abnormalities that occur when *Hsp83* activity is reduced, although at much greater frequencies than the abnormal traits observed among *Hsp83* hypomorph mutants, *Hsp83*<sup>e6D</sup> (Rutherford, and Lindquist, 1998; Sollars, Lu, *et al*, 2003). This increased penetrance is best illustrated by analyzing the notched wing phenotype, which occurs in approximately ~90% of iaRNA<sup>HSF</sup> expressing animals, but only present at ~5-20% in wild-type animals that have been raised in media containing the *Hsp83* inhibitor 17AAG during the first two generations after exposure. Surprisingly, less than 1% of *Hsp83*<sup>e6D</sup> mutants display this notched wing abnormality. Taken together, these results show that under non-heat shock conditions, iaRNA<sup>HSF</sup> expression compromises HSF ability to bind DNA, such as the *Hsp83* locus, and that this decrease in HSF occupancy results in decreased *Hsp83* transcript levels and mimicry of certain *Hsp83* mutant phenotypes.

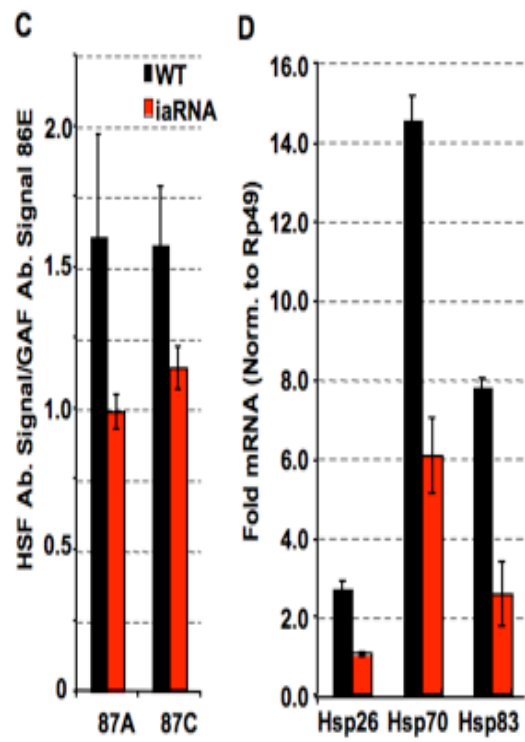
Under heat shock conditions, HSF becomes rapidly activated, resulting in the robust expression of heat shock genes (Saunders, Core, and Lis, 2006). However, high-level iaRNA<sup>HSFX48</sup> expression compromises HSF binding to the major *Hsp70* loci (87A and 87C) after exposure to heat stress (Figure 2.7B,

**Figure 2.7: Systemic iaRNA<sup>HSF</sup> expression inhibits HSF activity *in vivo*.**

A). HSF inhibition by iaRNA<sup>HSF</sup> results in adult animals that display abnormal animal morphology within abdominal segments, wings, thorax and eye structures at high frequencies (n>500 animals). B). High-level iaRNA<sup>HSF</sup> inhibits HSF binding to *Hsp70* genes under HS conditions. (Left column=WT, Right column=iaRNA<sup>HSFX48</sup>. Antibodies: Red=HSF, Green= GAF. Blue=DNA. Dot=87A locus, Triangle=87C locus). C). Quantification of the relative fluorescence of HSF in panel E at *Hsp70* (87AC loci) among WT or iaRNA<sup>HSFX48</sup> expressing animals (Signals normalized to GAF intensity at 86E locus. WT n=5; iaRNA<sup>HSF</sup> n=14). D). Constitutive iaRNA<sup>HSF</sup> expressing compromises major heat shock gene activation by HSF during heat stress (mRNAs quantified by RT-qPCR. Error % SEM, WT n=4, iaRNA<sup>HSF</sup> n=4) (Figure is displayed on multiple pages)







left column=WT, right column=iaRNA<sup>HSF</sup> expressing animals). Quantification of the HSF antibody signals normalized to GAGA factor antibody staining at a nearby site that does not undergo “puffing” (86E) shows the strong level of iaRNA<sup>HSF</sup> inhibition of the ability of HSF to bind at both the 87A and 87C loci (Figure 2.7C). Furthermore, quantification of the total RNA from three major classes of heat shock gene, *Hsp26*, *Hsp70* and *Hsp83*, demonstrates that iaRNA<sup>HSF</sup> expression compromises overall HSF activity and thus attenuates the normal heat shock response (Figure 2.7D). Figures 2.6 and 2.7 demonstrate that iaRNA<sup>HSF</sup> expression results in decreased HSF activity under both normal and heat-shock conditions *in vivo*.

### **2.2.3. Functional specificity of the *in vivo* iaRNA<sup>HSF</sup>-HSF interaction**

Our engineered molecule, iaRNA<sup>HSF</sup>, shows several properties of a highly specific drug, including its high affinity for HSF (5-10 nM *in vitro*) and its ability to inhibit HSF binding to target genes as well as the expression of heat shock genes *in vivo*. To more rigorously prove the specificity of the iaRNA<sup>HSF</sup> molecule to HSF, I made use of the fact that over-expression of either iaRNA<sup>HSF</sup> or HSF results in increased lethality during the pupae stage. I reasoned that this lethality could be ameliorated by overexpressing the complementary interacting partner within the same organism. This genetic approach is analogous to factor titration or add-back *in vitro* binding assays, where the effects of an RNA aptamer are reversed by the addition of excess target protein (Sevilimedu, Shi, and Lis, 2008; Shi, Hoffman, and Lis, 1999; Zhao, Shi, *et al*, 2006).

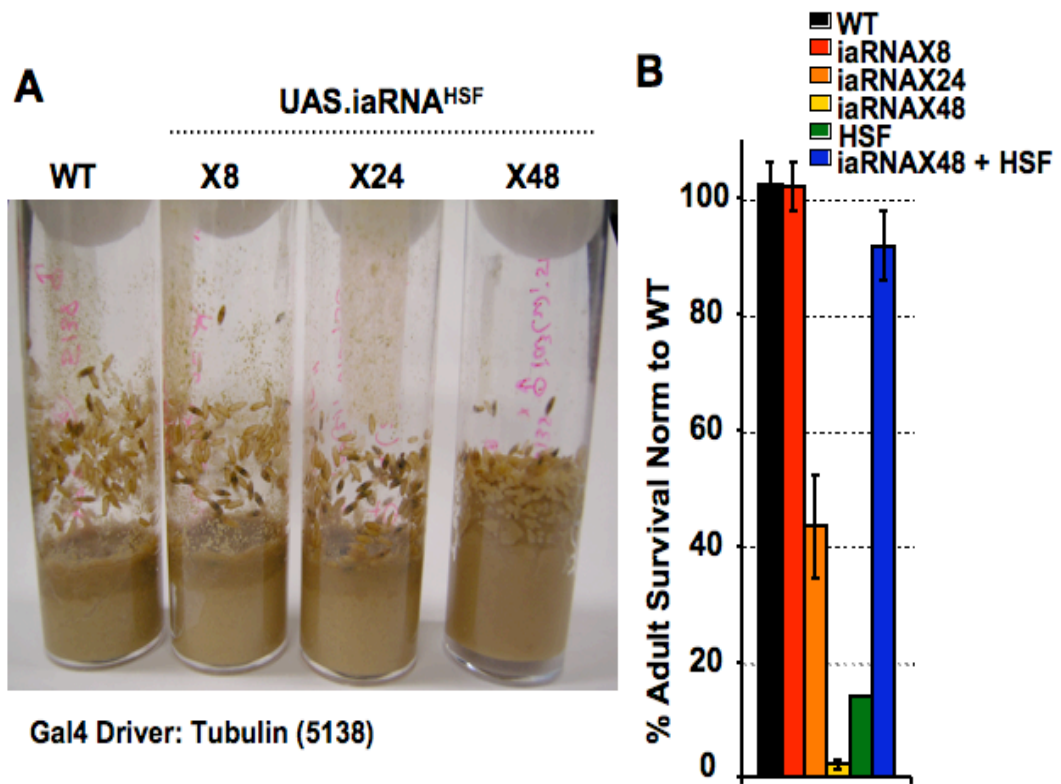
In the first scenario, I asked if HSF over-expression could suppress the abnormalities induced by iaRNA<sup>HSF</sup> over-expression. Here, systemic

iaRNA<sup>HSF</sup> expression results in lethality that occurs with increasing iaRNA<sup>HSF</sup> gene dosage (Figure 2.8A compare animals that express 8, 24 and 48 iaRNA<sup>HSF</sup> genes), a finding that could potentially be attributed to the fact that HSF is an essential gene in *Drosophila* (Jedlicka, Mortin, and Wu, 1997). This iaRNA<sup>HSF</sup>-induced effect is effectively suppressed upon HSF coexpression (Figure 2.8B, compare gray and blue column). Additionally, I took advantage of *Drosophila* genetics to ask if the abnormal notching wing defect that occurs at high frequency in iaRNA<sup>HSF</sup> expressing animals could be suppressed by co-expression of a non-iaRNA<sup>HSF</sup> binding protein such as GFP. Figure 2.8C shows that the notched wing defect only occurs in iaRNA<sup>HSF</sup>-expressing animals and is absent in any of the parental stocks (compare parental controls i-ii versus iii). This abnormality is not altered upon GFP over-expression but is effectively rescued upon overexpression of either HSF, or Hsp83, a major product of HSF activity in non-stressed cells (Figure 2.8C, compare iv versus v & vi respectively).

I also assayed if iaRNA<sup>HSF</sup> over-expression could ameliorate the abnormalities induced by HSF over-expression through the use of a complementarity test. Here, I find that tissue-specific HSF over-expression results in abnormally small salivary glands in *Drosophila* (Figure 2.8B, compare right and left panel), and that this abnormality is suppressed when HSF is coexpressed with iaRNA<sup>HSF</sup> (Figure 2.8D, middle panel). Comparison of the salivary gland length between *Drosophila* and strains that over-express HSF or animals that over-express HSF and iaRNA<sup>HSF</sup> shows that iaRNA<sup>HSF</sup> can inhibit HSF *in vivo* and restore the salivary gland morphology to near wild type size (Figure 2.8D). Furthermore, I find that high-level systemic HSF over-expression also results in a high frequency of lethality (Figure 2.8B) that is

**Figure 2.8: iaRNA<sup>HSF</sup> is specific to HSF *in vivo*.** A). Aptamer expression induces lethality in a gene dosage dependent manner. (WT is parental Gal4 line; X8, X24, X48 corresponds to aptamer gene dosage under tubulin Gal4 transcriptional control). B). Co-expression of iaRNA<sup>HSF</sup> and HSF suppress the lethality's induced by either high-level iaRNA<sup>HSF</sup> or HSF over-expression (Error= number of surviving adults over 200 *Drosophila* larvae). C). HSF and Hsp83 over-expression, but not GFP over-expression suppresses the notched wing phenotype induced by high-level iaRNA<sup>HSF</sup> expression (I & II are WT parental controls: UAS.iaRNA<sup>HSF</sup> & Tubulin Gal4 stocks). (III) iaRNA<sup>HSF</sup> expressing animals with notched wings (UAS.iaRNA<sup>HSF</sup> + Tubulin Gal4). (IV) iaRNA<sup>HSF</sup> expressing animals that over-express GFP protein (UAS.GFP+UAS.iaRNA<sup>HSF</sup>+TubGal4). (V) iaRNA<sup>HSF</sup> expressing animals with an additional copy of *Hsp83* gene (UAS.iaRNA<sup>HSF</sup> + Tubulin Gal4 + duplication 61F-64B); and (VI) corresponds to animals that over-express iaRNA<sup>HSF</sup> and HSF protein (UAS.iaRNA+Tubulin Gal4+UAS.HSF). D). iaRNA<sup>HSF</sup> suppresses the adverse effects of HSF over-expression in the *Drosophila* salivary glands (Left=over-express HSF<sup>GFP</sup>; Middle= over-express HSF<sup>GFP</sup>+iaRNA; Right=WT. Salivary gland Gal4 driver 6983, p{GawB}c729). E). Quantification of salivary gland length of panel C (Error % SEM, WT n=9; HSF<sup>GFP</sup> n=8; HSF<sup>GFP</sup>+iaRNA<sup>HSF</sup> n=15) (Figure is displayed on multiple pages).





**Figure 2.8: (Continued)**

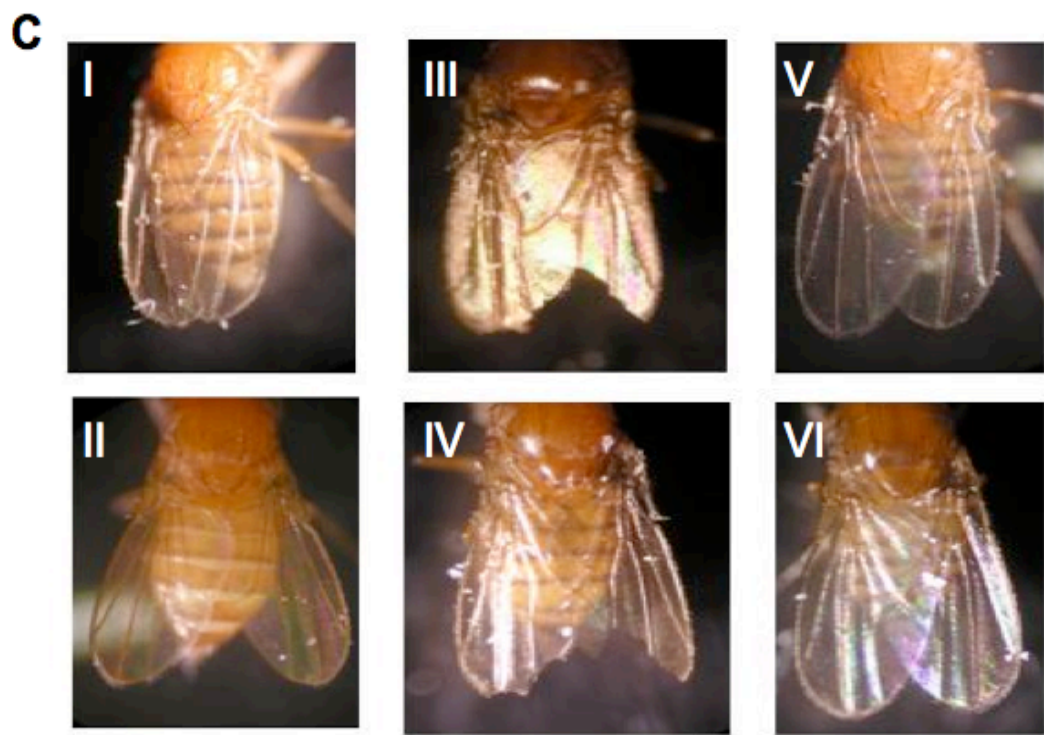
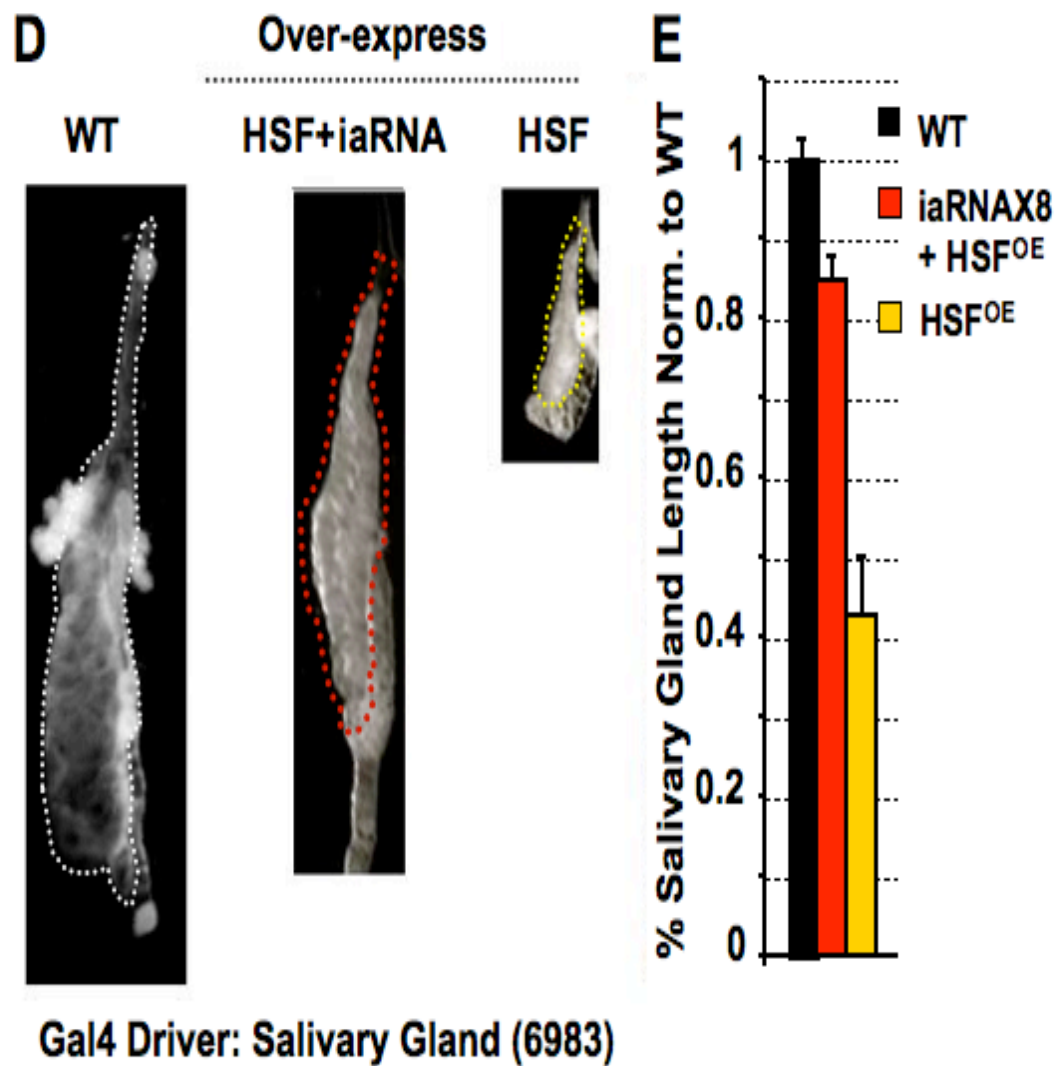


Figure 2.8 (Continued)



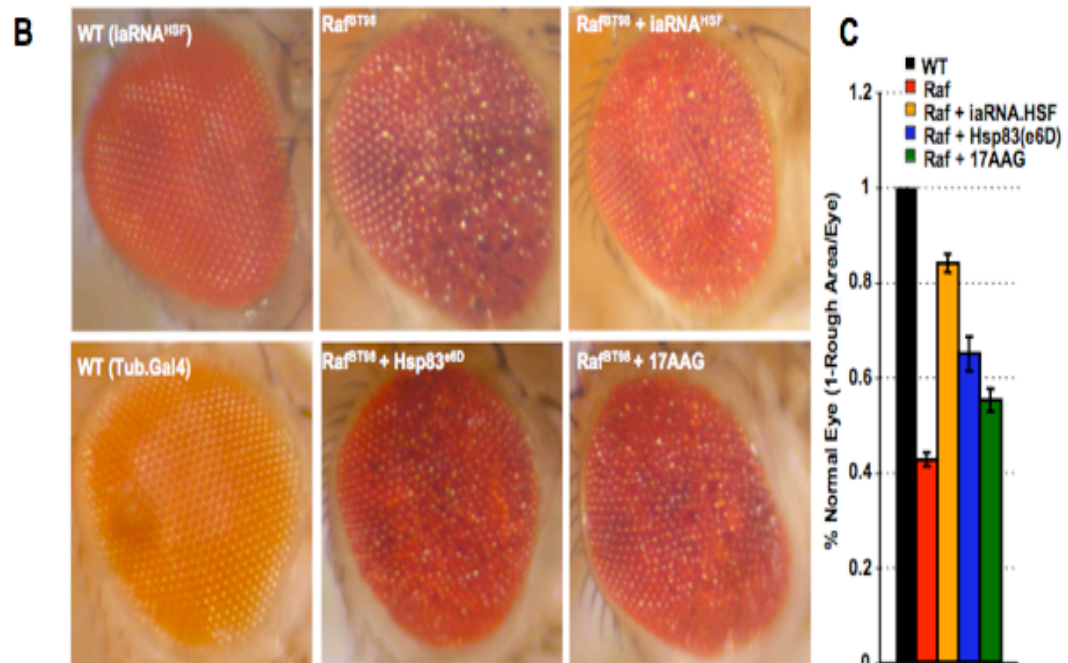
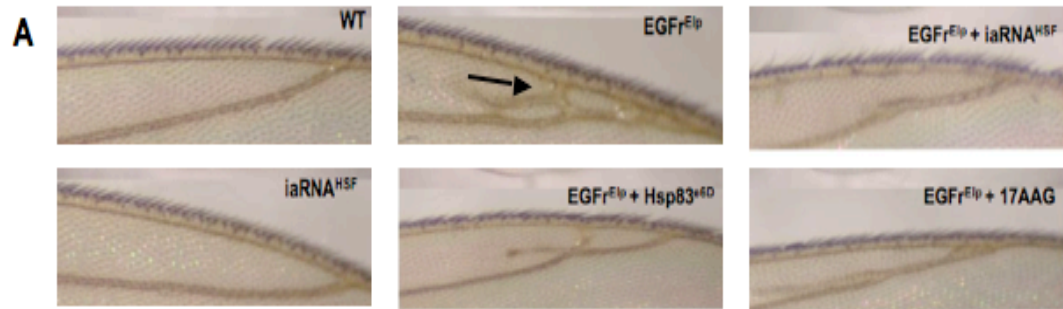
effectively suppressed by iaRNA<sup>HSF</sup> co-expression, resulting in viable and fertile animals (Figure 2.8B). Taken together, I conclude that the iaRNA<sup>HSF</sup> does not produce its phenotypes by binding to random proteins, but rather acts on the intended target, HSF, by inhibiting expression of HSF's primary target, Hsp83, under non-inducing conditions.

#### **2.2.4 iaRNA<sup>HSF</sup> expression attenuates phenotypes of hyperactive mutations in the *Drosophila* MAPK signaling pathway**

As mentioned previously in chapter 1, Hsp83 (Hsp90 in mammals) is known to modulate the MAPK signaling pathway, a well conserved and important regulatory pathway that is commonly overactivated in human tumors (Jones, Zhang, *et al*, 2008; Parsons, Jones, *et al*, 2008; Pratt, and Toft, 2003). In *Drosophila*, gain-of-function mutations within the MAPK pathway do not result in tumor formation; rather, hyperactivation of the MAPK pathway results in altered cell fate specification and abnormal tissue morphology. Components of this pathway, such as the epidermal growth factor receptor (EGFR) and Raf oncogenes, depend on normal levels of *Hsp83* activity (Biggs, Zavitz, *et al*, 1994; Citri, Gan, *et al*, 2004; Neckers, 2002; Sawai, Chandarlapaty, *et al*, 2008; Van der Straten, Rommel, *et al*, 1997). In these cases, Hsp83 is required for maintaining these signaling proteins in a properly folded state as well as localizing and regulating their kinase activity. This dependency by MAPK components on Hsp83 activity encouraged me to investigate the inhibitory potential of the HSF aptamer *in vivo* using *Drosophila* gain-of-function EGFR<sup>Eip</sup> or Raf<sup>BT98</sup> mutants given my previous work where I found that HSF inhibition reduces the total levels of Hsp83 protein.

First, I asked whether  $\text{iaRNA}^{\text{HSF}}$  expression could effectively suppress the abnormalities induced by gain-of-function mutations of the *Drosophila*  $\text{EGFR}^{\text{Eip}}$  and  $\text{Raf}^{\text{BT98}}$  oncogenes. In the former case, heterozygous flies that express  $\text{EGFR}^{\text{Eip}}$  have been previously shown to contain abnormal wing vein morphology (Li, and Li, 2003) (Figure 2.9A). This abnormality is effectively suppressed when HSF is inhibited by  $\text{iaRNA}^{\text{HSF}}$ , when Hsp83 activity is compromised by the expression of the  $\text{Hsp83}^{\text{e6D}}$  antimorphic mutant, or when hemizygous  $\text{EGFR}^{\text{Eip}}$  flies are treated with 17-AAG (Figure 2.9A). Similarly, heterozygote animals that express gain-of-function  $\text{Raf}^{\text{BT98}}$  have been shown to contain multiple R7 cells within each ommatidium, resulting in flies with a rough eye morphology (Van der Straten, Rommel, *et al*, 1997) (Figure 2.9B) that has been shown to be dependent of Hsp83 activity (Van der Straten, Rommel, *et al*, 1997). Here, I show that this phenotype is also effectively suppressed by  $\text{iaRNA}^{\text{HSF}}$  expression, by reducing Hsp83 activity by the expression of the  $\text{Hsp83}^{\text{e6D}}$  antimorphic mutant, or treatment of  $\text{Raf}^{\text{BT98}}$  animals with 17-AAG (Figure 2.9B). Approximation of the affected surface area of the eye in each of these genetic backgrounds shows that overactive MAPK signaling phenotypes causes a rough eye phenotype and this abnormality can be effectively attenuated by  $\text{iaRNA}^{\text{HSF}}$  expression or Hsp83 inhibition (Figure 2.9C). This reversal of the tumor-related phenotypes in *Drosophila* caused by known oncogenes encouraged me to extend my analysis of the HSF aptamer's potential anti-tumor properties to mammalian cells.

**Figure 2.9: Expression of iaRNA<sup>HSF</sup> suppresses gain-of-function mutations of genes in the MAPK signaling pathway.** A). iaRNA<sup>HSF</sup> expression and Hsp83 inhibition suppress the abnormal wing phenotype induced by the EGFr<sup>Elp</sup> mutant. B). iaRNA<sup>HSF</sup> expression and Hsp83 inhibition suppress the rough eye phenotype induced by activated Raf<sup>BT98</sup> mutant. C). Quantification of rough eye phenotypes in panel B calculated by area. Error % SEM, WT (n=5); Raf<sup>BT98</sup> (n=26); Raf<sup>BT98</sup> + iaRNA<sup>HSF</sup> (n=33); Raf<sup>BT98</sup> + Hsp83<sup>e6D</sup> (n=33); Raf<sup>BT98</sup> + 3.6uM 17AAG (n=33).



### **2.2.5. iaRNA<sup>HSF</sup> expression induces apoptosis in human cancer cells**

To investigate the effects of iaRNA<sup>HSF</sup> in human cancer cells, I expressed the aptamer in cervical carcinoma (HeLa), and chemically transformed kidney cells (293T) and determined its effects within the first 5 days of expression by culturing the cells using the selectable marker blasticidin, which is encoded within the vector containing the aptamer. Non-transfected cells were killed within the first day using a predetermined amount of blasticidin, and only those cells that were resistant to blasticidin treatment were used throughout the rest of the experiments. Among transfected cells, iaRNA<sup>HSF</sup> expression dramatically altered the morphology of cells causing them to round up and detach from the culture plate. Interestingly, iaRNA<sup>HSF</sup> expression did not have significant effects on the morphology of primary non-transformed human cells (IMR90) (Figure 2.10A). To ensure that both the primary (IMR90) and cancerous cells express aptamer RNA to a similar extent, I performed quantitative real-time PCR (qPCR) and found that the relative aptamer levels were approximately equal for both control and the HSF aptamer RNA in both cell lines (Figure 2.10B). Because detached cells often correlate with increased cell death, I performed apoptotic assays in collaboration with Marc Antonyak in Rick Cerione's laboratory. Together, we stained cells with DAPI and quantified the number of apoptotic cells in parental cells that either lacked, or expressed the HSF aptamer or a control RNA (Rev) using nuclear fragmentation as a read-out of apoptosis (Figure 2.10C). We found that iaRNA<sup>HSF</sup> expression did not result in a significant amount of cell death in the normal IMR90 cells. In contrast, iaRNA<sup>HSF</sup> expression induced an approximately 9-fold increase in cell death among HeLa cells (63% of the population,  $p=0.0001$ ), and approximately a 7-fold increase in apoptosis in



**Figure 2.10: iaRNA<sup>HSF</sup> induces a cancer cell specific apoptotic response.**

A). iaRNA<sup>HSF</sup> expression induces a morphological defect among transformed human cervical carcinoma (HeLa) and chemically transformed human kidney cells (293T); but not in a primary human lung fibroblast cell line (IMR90) after 96 hours post transfection. B). Control RNA and aptamer RNA (iaRNA<sup>HSF</sup>) are expressed to near equal levels in semi-stable HeLa & IMR90 cells after 24hrs of RNA expression (RNA values normalized to GAPDH, n=3). C). Expression of iaRNA<sup>HSF</sup> induces apoptosis in HeLa and 293T cells approximately ~10-fold (p<0.0001), and ~7-fold (p<0.0001) respectively (apoptosis was determined by quantifying the number of cells undergoing nuclear fragmentation scored by DNA staining at 96hrs post RNA expression, all values n>8) (Figure is displayed on multiple pages).

**A**

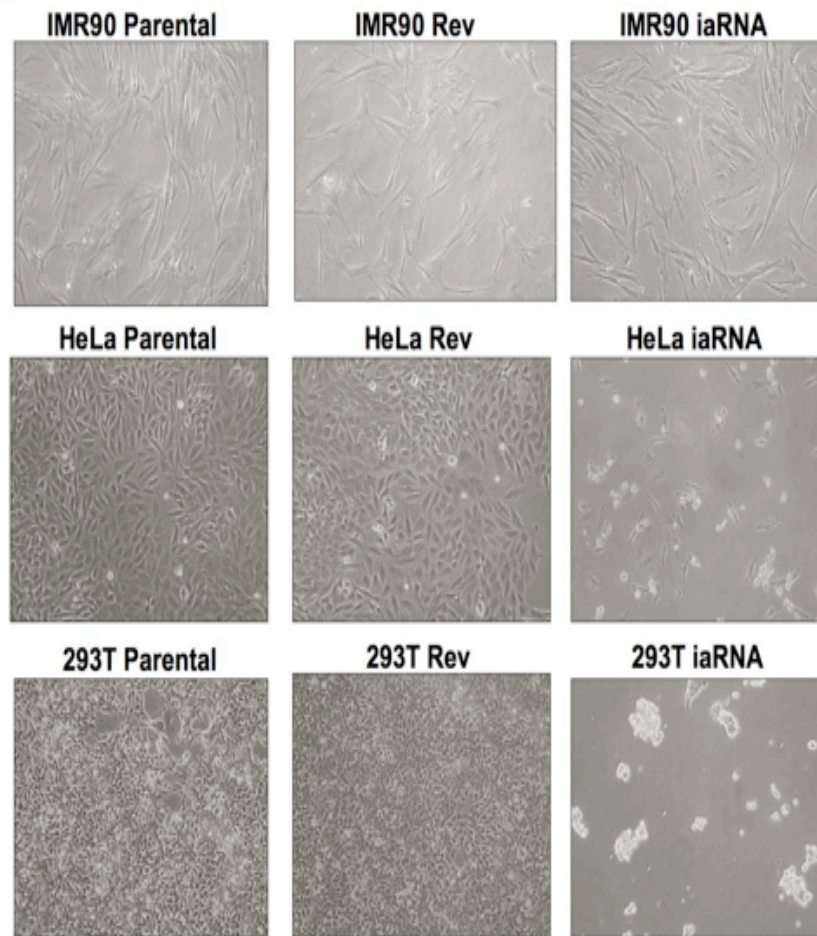
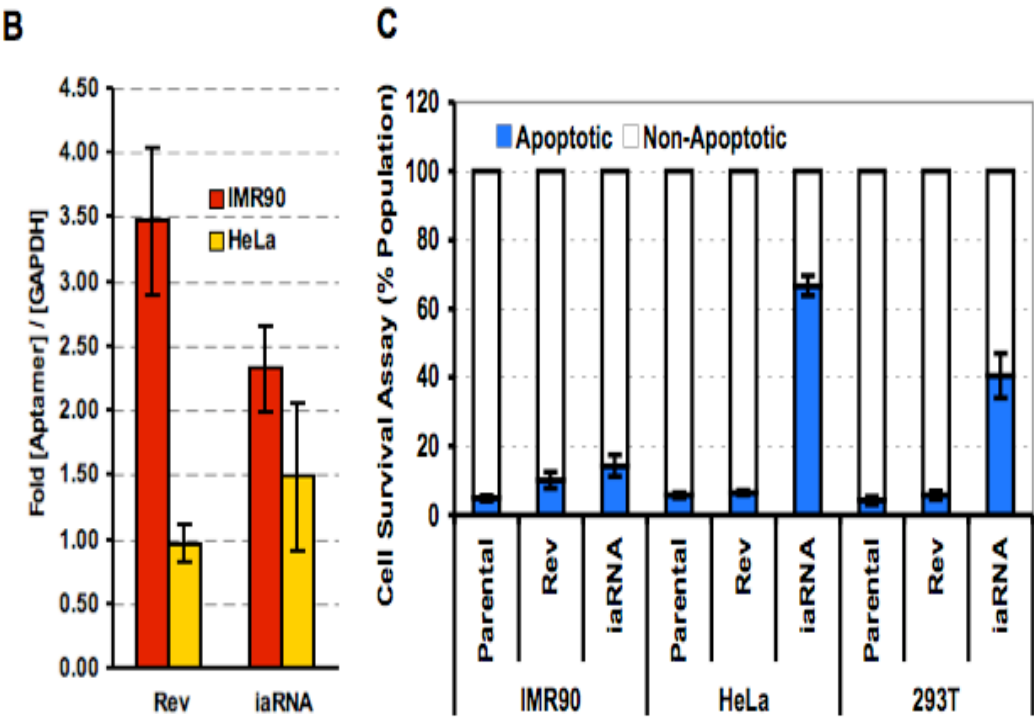


Figure 2.10 (Continued)



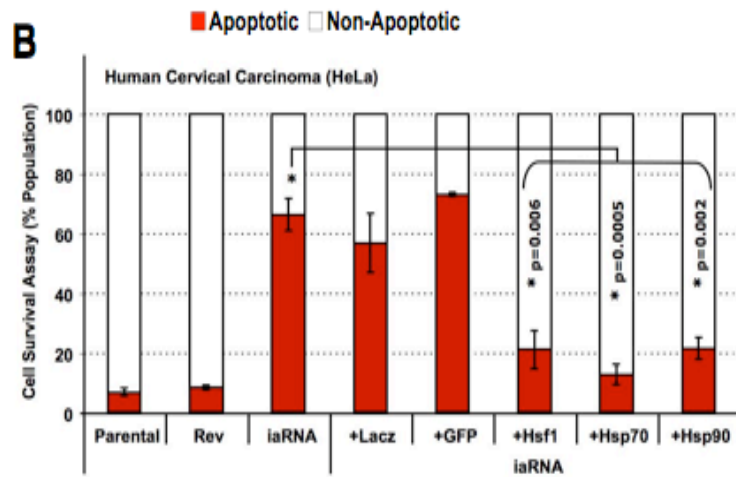
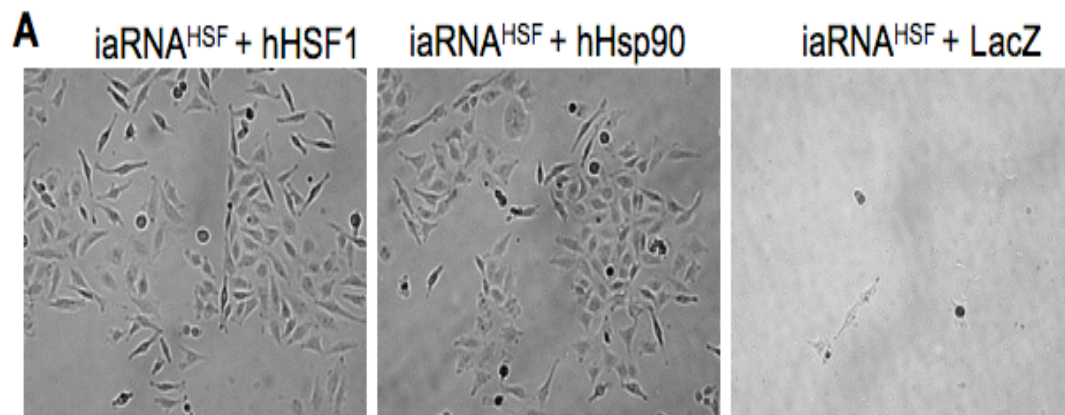
\*Cells visualized and apoptosis was quantified ~96hrs post aptamer expression

293T cells (40% of the population,  $p=0.0018$ ) relative to cancer cells expressing a control RNA. Collectively, our results indicate that HSF1 inhibition results in a cancer cell-specific apoptotic response.

Results in *Drosophila* revealed an HSF-Hsp90 regulatory interaction wherein HSF inhibition results in reduced *Hsp90* expression, and the presentation of *Hsp90* mutant-like phenotypes. By contrast, *Hsp90* over-expression attenuates the abnormalities induced by high-level expression the HSF aptamer. To explore whether this relationship also extends to cancer cell survival, I examined whether over-expression of *Hsp90* suppresses the apoptotic response induced by  $\text{iaRNA}^{\text{HSF}}$  expression in HeLa cells (Figure 2.11A). Indeed, I found that *Hsp90*, *Hsp70* or *HSF1* over-expression all effectively suppress the apoptotic response, indicating that HSF1 inhibition compromises the activity of the Hsp90/Hsp70 multi-chaperone complex (Figure 2.11B).

Because approximately ~30% of  $\text{iaRNA}^{\text{HSF}}$ -treated HeLa cells do not undergo apoptosis in our time restricted assays, I sought to analyze the prolonged effects of  $\text{iaRNA}^{\text{HSF}}$  expression using anchorage-independent soft agar growth assays as an *in vitro* measurement of tumorigenicity. As expected, HeLa cells expressing the Rev control RNA sequence grew in the soft agar matrix and formed large colonies to the same extent as non-transfected control cells (Figure 2.12); however, HeLa cells expressing  $\text{iaRNA}^{\text{HSF}}$  do not. This shows that those cells that express  $\text{iaRNA}^{\text{HSF}}$  yet have not undergone apoptosis (Figure 2.10C) exhibit a compromised transformation capacity that results in reduced growth on soft agar.

**Figure 2.11: Suppression of iaRNA<sup>HSF</sup> induced apoptosis by over-expression of molecular chaperones.** A). Over-expression of HSF1 or Hsp90, but not LacZ rescues the apoptotic response induced by iaRNA<sup>HSF</sup> over-expression. B). Quantification of results in panel A shows that over-expression of molecular chaperones (HSF1  $p<0.006$ , Hsp90  $p<0.005$ , or Hsp70  $p<0.002$ ), but not random proteins (GFP or LacZ) can effectively rescue the aptamer induced apoptotic response (apoptosis was determined by quantifying the number of cells undergoing nuclear fragmentation scored by DNA staining at 96hrs post RNA expression, all values  $n>8$ ).



Having established that Hsp90 over-expression rescues the aptamer effects within cervical carcinoma cells (Figure 2.11), and that treatment of HeLa cells with Hsp90 inhibitors compromises cancer cell viability (Bisht, Bradbury, *et al*, 2003), I decided to compare the inhibitory effects of iaRNA<sup>HSF</sup> expression with 17-AAG treatment using colony formation as a readout. In this experiment, iaRNA<sup>HSF</sup> expression induces a similar cytotoxic response in HeLa cells relative to the treatment of this cell type to 150nM 17-AAG (Figure 2.12, right panels). Overall, HSF1 inhibition by iaRNA<sup>HSF</sup> closely resembles the effects observed upon Hsp90 inhibition in both *Drosophila* and human cancer models (Figures 2.9- 2.12).

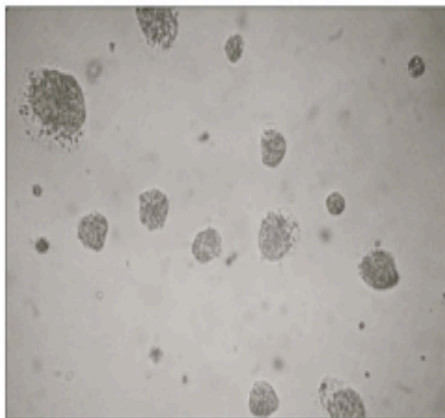
#### **2.2.6. HSF regulates cancer cell survival by compromising the activity of members of a chaperone ‘buffering’ network**

To further explore the role of HSF1 inhibition and the resulting reduction in Hsp90 activity in human cancer cells, I examined the effects of iaRNA<sup>HSF</sup> on the MAP kinase pathway, which is known to be dependent on Hsp90 activity (Figure 2.9) (Bisht, Bradbury, *et al*, 2003; Pratt, and Toft, 2003; Cancer Genome Atlas Research Network, 2008). I used EGF to activate this pathway in parental HeLa cells as well as HeLa cells expressing aptamer or control RNA (Figure 2.13). By measuring the effects of EGF stimulation under conditions prior to the onset of apoptosis, I investigated the primary role of HSF1 inhibition on this specific cell-survival pathway without the complication of proteolysis induced by the apoptotic response. In these experiments, MAPK pathway activation was measured using the Erk-p T202/Y204 antibody that recognizes the activated form of Erk1/2. Here, I find that parental or control RNA expressing HeLa cells contain high levels of total Erk1/2 protein

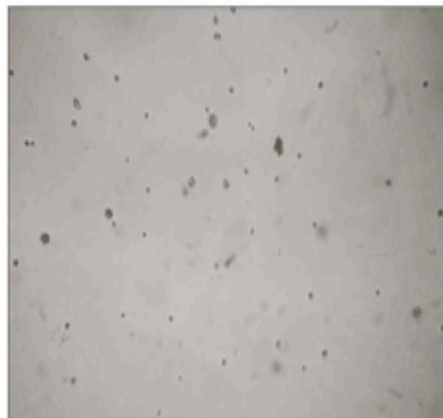
**Figure 2.12: HSF1 inhibition by iaRNA<sup>HSF</sup> inhibits transformed growth in soft agar.** Soft agar analysis of non-transfected HeLa cells (top left), control RNA over-expressing HeLa (bottom left), shows that iaRNA<sup>HSF</sup> over-expression (bottom right) inhibits cellular transformation (colony formation) in a similar manner as treatment of HeLa cells with 150nM 17-AAG (top right), a potent anti-cancerous pharmacological agent (pictures taken during Day 14).



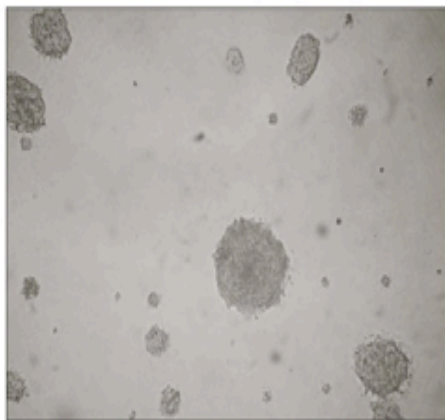
**Hela Parental (Day 14)**



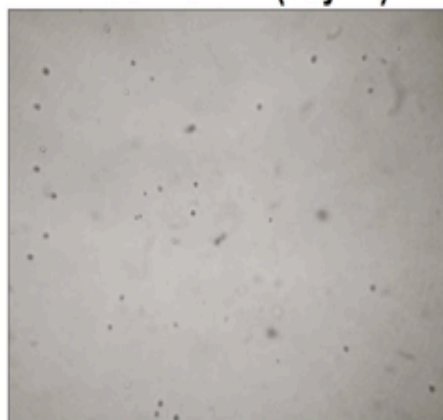
**Hela + 17AAG (Day 14)**



**Hela + Rev (Day 14)**

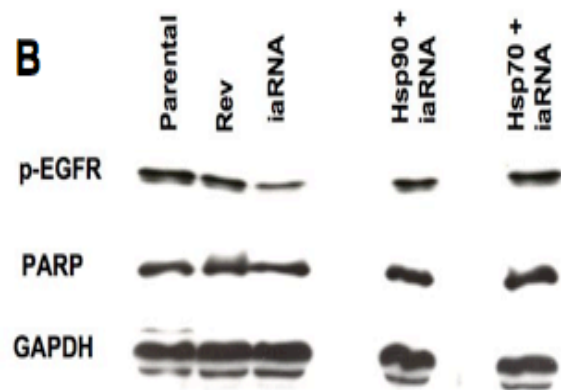
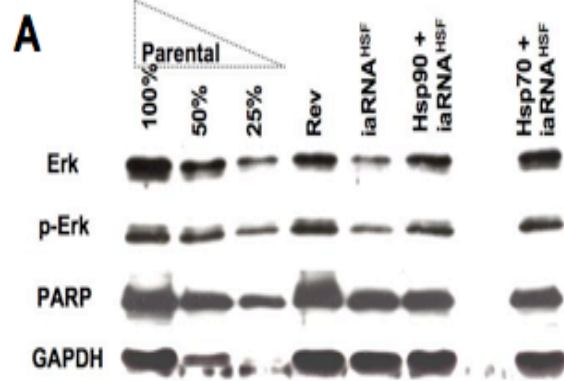


**Hela + iaRNA (Day 14)**

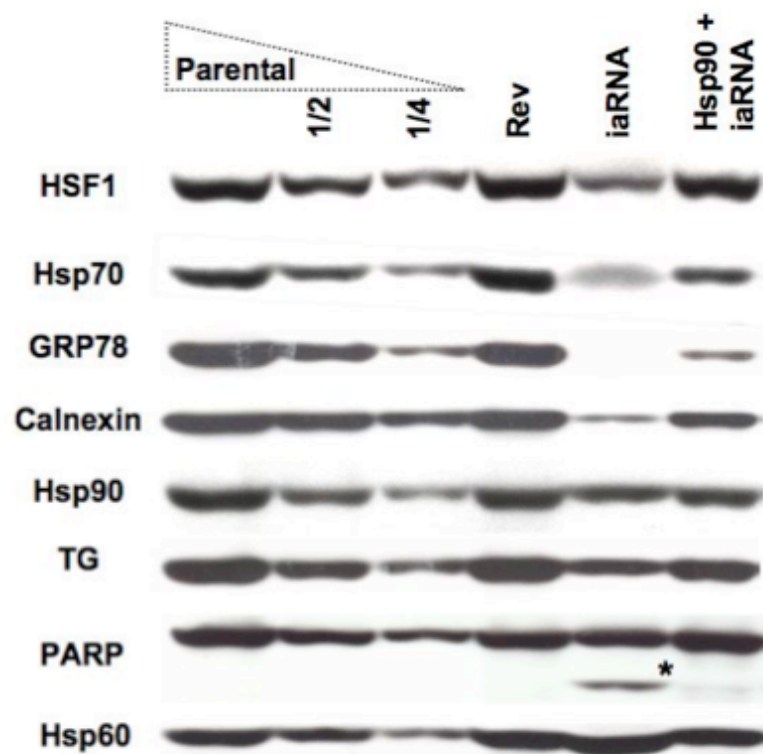


that becomes activated upon EGF treatment (Erk-p) (Figure 2.13A). In contrast, HSF1 inhibition by the aptamer results in a >75% decrease in total Erk1/2 levels, and this decreased level of Erk1/2 protein results in a corresponding decrease in the activated form of the protein (Figure 2.13A). Furthermore, I measured the levels of both total and phosphorylated Erk1/2 protein in aptamer expressing cells that either over-express Hsp90 or its co-chaperone Hsp70 partner, since the latter protein has also been shown to work in conjunction with Hsp90 in a multi-protein chaperone complex required by cancer cells for survival (Bagatell, and Whitesell, 2004; Workman, 2004; Powers, Clarke, and Workman, 2008;). Here, over-expression of either Hsp90 or Hsp70 rescues the loss of Erk1/2 protein, resulting in normal MAPK signaling activity. This observation suggests that the attenuation of the MAPK signaling pathway that occurs upon HSF1 inhibition is due, at least in part, to downregulation of Hsp90 activity. Using an antibody that recognizes the activated form of the EGF receptor (EGFR-p Y1068) I determined that aptamer-expressing HeLa cells contained decreased levels of activated forms of the EGF receptor after ligand treatment and that this effect was restored by the over-expression of Hsp70 or Hsp90 protein (Figure 2.13B). I next considered whether the effects of HSF1 inhibition on cancer cells is due solely to the moderate depletion of Hsp90 levels, or whether apoptosis was triggered by the concerted depletion of other molecular chaperones. Strikingly, I found that HSF1 inhibition by iaRNA<sup>HSF</sup> resulted in a severe reduction of Hsp70 levels (Figure 2.14). This finding suggests that the anti-cancerous properties of the aptamer are due in part to the restoration of pro-apoptotic signaling events, since Hsp70 directly inhibits the recruitment of pro-caspase 9 to the apoptosome (Beere, Wolf, *et al*, 2000; Jaattela, Wissing, *et al*, 1998; Steel,

**Figure 2.13: HSF1 inhibition by iaRNA<sup>HSF</sup> compromises MAPK signaling in human cancer cells.** A). iaRNA<sup>HSF</sup> expression decreases the total levels of Erk protein resulting in a similar decrease in the levels of Erk phosphorylation upon activation of MAPK by EGF addition (MAPK activation was induced by conditioning HeLa cells in serum free media for 12hrs before the addition of 100ng/ml EGF for 10 min. *For more details see Chapter 4-Methods*). Intriguingly, the aptamer induced Erk loss is effectively rescued when molecular chaperones (Hsp90 & Hsp70) are over-expressed. B). Similarly, iaRNA<sup>HSF</sup> inhibits EGFR activation (EGFR~p) upon MAPK activation by EGF ligand treatment (same conditions as panel A), and this effect is rescued by Hsp90 or Hsp70 over-expression. (Triangles represent serial dilutions of parental (non-transfected) HeLa cells to be used for quantification purposes. In these experiments, whole cell extracts were isolated from samples NOT undergoing apoptosis as determined by both cytological inspection before harvesting, and by the intact 'non-cleaved' PARP antibody signal observed after immunoblotting. Western blots contain ~40ug of total protein per sample electrophoresed on 10% SDS denaturing gels blocked with 10% BSA instead of 5% milk).



**Figure 2.14: HSF1 inhibition by iaRNA<sup>HSF</sup> reduces levels of members of the ‘chaperone network’.** Determination of the steady state levels of various molecular chaperones shows that HSF1 inhibition by iaRNA<sup>HSF</sup> results in a dramatic decrease among certain molecular chaperones in human cervical carcinomas cell compared with non-transfected parental cells, control RNA expressing (Rev), or cells that over-express iaRNA<sup>HSF</sup> & Hsp90. Note that iaRNA<sup>HSF</sup> triggers an apoptotic response as shown by the cleaved PARP product\*. In these experiments Hsp60 was used as a loading control because its proteins levels did not seem to change throughout the course of the experiment.



Doherty, *et al*, 2004). The anti-apoptotic functions of Hsp70 are emphasized by the fact that Hsp70 is expressed at high levels in many cancer types (Becker, Multhoff, *et al*, 2004; Nanbu, Konishi, *et al*, 1998) as well as by our findings that demonstrate that Hsp70 over-expression effectively attenuates the apoptotic response induced by the aptamer (Figure 2.14). The above findings suggest that attenuating apoptosis by Hsp70 over-expression can also restore pro-survival signaling pathways (MAPK) in HeLa cells. In any event, prolonged high-level aptamer expression clearly induces apoptosis as demonstrated by probing for the loss and cleavage of the well-documented caspase-3 downstream target, PARP (Figure 2.14). Under these same conditions, we notice that the levels of HSF1 and various other chaperones, including calnexin, PDI and Grp78 are depleted (Figure 2.14). Hsp60, a mitochondrial specific chaperone, does not seem to be affected upon aptamer expression, suggesting that Hsp60 is either a highly stable protein or that its expression is independent of HSF1 transcriptional activity.

To address the possibility that the loss of Hsp70, GRP78, and calnexin observed upon aptamer expression is not due to apoptosis-induced proteolysis, but rather by the inhibition of HSF1 transcriptional stimulatory activity, I determined the levels of these proteins under conditions where apoptosis was inhibited by Hsp90 co-expression (Figure 2.13 -2.14). Under these conditions, I found that total HSF1 levels remain normal, indicating that the loss of HSF1 protein observed upon aptamer expression is not due to aptamer binding itself and causing degradation of the protein, but rather due to apoptosis. However, these cells still have reduced amounts of Hsp70 (~50%), Grp78 (~25%), and calnexin (~50%) relative to (non-transfected)

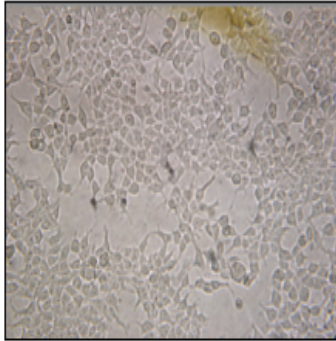
HeLa cells, or RNA expressing control cells. This result indicates that these chaperones are HSF1 regulated genes.

This simplest model to account for these findings is that the HSF aptamer acts by preventing HSF1 from binding to the heat shock elements on the promoters of the chaperone genes. HSF1 is known to regulate the expression of many chaperones in mammals (Murray, Whitfield, *et al*, 2004; Trinklein, Murray, *et al*, 2004), some of which are therapeutic markers in the prognosis of diseases like cancer (Becker, Multhoff, *et al*, 2004; Nanbu, Konishi, *et al*, 1998). I therefore decided to determine how iaRNA<sup>HSF</sup> expression affects highly aggressive cancers that depend largely on normal Hsp90 activity and Hsp90 regulated signaling pathways (Pratt, and Toft, 2003). Here I found that the expression of iaRNA<sup>HSF</sup> significantly disrupts the cellular morphology of MDA-MB-231 highly aggressive breast cancer cells or BE(2)-M17 brain glioblastoma cells (Figure 2.15) in a similar manner as the expression seen in cervical carcinomas (Figure 2.10A). This evidence supports the idea that the anti-cancerous properties of iaRNA<sup>HSF</sup> are due to the depletion of specific members of a larger chaperone network that is critical for maintenance of the cellular integrity of transformed cells.

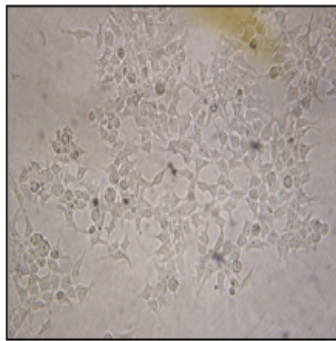


**Figure 2.15: HSF1 inhibition by iaRNA<sup>HSF</sup> compromises survival of highly aggressive breast and brain cancers.** iaRNA<sup>HSF</sup> expression induces a morphological defect among human breast cancer cells (MDA-MB-231) and human brain glioblastoma cells (BE(2)-M17). In these experiments, each cell line was transfected with a mock vector, reverse aptamer sequence or the HSF1 aptamer and expressed using the EF1-a highly constitutive promoter. Semistable cell lines were analyzed after approximately 1 week post transfection and selection using the minimal amount of selection marker as determined by kill curve experiments (1ug/ml blasticidin).

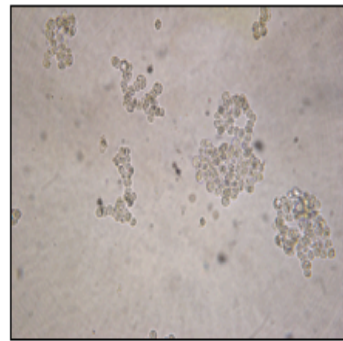
**MDA-MB-231 Parental**



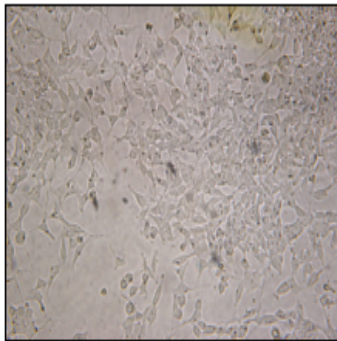
**MDA-MB-231 Rev**



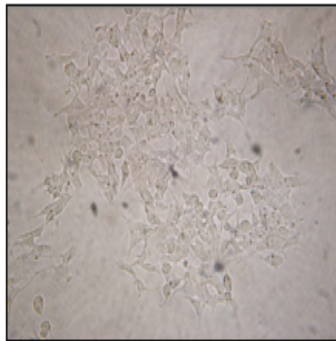
**MDA-MB-231 iaRNA**



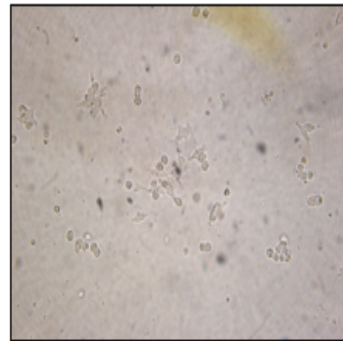
**BE(2)-M17 Parental**



**BE(2)-M17 Rev**



**BE(2)-M17 iaRNA**



## **2.3. SUMMARY AND DISCUSSION**

### **2.3.1. Stress response, molecular chaperones and tumorigenesis**

Cancer is a disease of genetic instability that promotes cellular adaptation to adverse microenvironments through specific mutations in proteins that promote deregulated cell growth (Hanahan, and Weinberg, 2000; Stoler, Chen, *et al*, 1999). Because cancer cells generally exhibit increased proliferation rates compared to non-transformed cells, cancer cells require higher levels of nutrients, and therefore exhibit increased metabolic processes in poorly oxygenated, or hypoxic, microenvironments that result in highly-stressed growth conditions (Hahn, and Weinberg, 2002; Jolly, and Morimoto, 2000). Therefore, in order for cancer cells to tolerate the higher levels of free radicals and the increased proteotoxicity generated by undergoing such adaptation, transformed cells generally increase the expression levels of molecular chaperones that can overcome the protein damage caused by this stressful environment. Historically, the cytoprotective function of molecular chaperones was originally studied using thermotolerance as a model. Here, animals containing elevated amounts of chaperones induced by a mild pre-heat treatment can survive extreme stress conditions that are otherwise lethal in that particular organism (Sanchez, and Lindquist, 1990; Parsell, Sanchez, *et al*, 1991; Parsell, and Lindquist, 1993; Sanchez, Parsell, *et al*, 1993).

Recently, strong evidence suggests that HSF1 activity is critical for cellular transformation and adaptation of cells to microenvironments that demand higher chaperone activity such as cancers (Dai, Whitesell, *et al*, 2007). In this regard, molecular chaperones that are regulated by HSF1 have been shown to stabilize the folding of mutated proteins such as dominant negative forms of the p53 tumor suppressor, various hormone regulated

receptors, and non-receptor tyrosine kinase (Blagosklonny, Toretsky, *et al*, 1996; Cancer Genome Atlas Research Network, 2008). Therefore, it is likely that many of the mutated proteins that promote carcinogenesis are stabilized by molecular chaperones (Blagosklonny, Toretsky, *et al*, 1996; Pinhasi-Kimhi, Michalovitz, *et al*, 1986; Luo, Solimini, *et al*, 2009). The fact that cancer cells show increased levels of heat shock proteins (Hsps), in particular Hsp70 and Hsp90 (Becker, Multhoff, *et al*, 2004; Nanbu, Konishi, *et al*, 1998), together with the fact that the inhibition of these proteins sensitizes cancer cells to pharmacological treatment (Basso, Solit, *et al*, 2002; Bisht, Bradbury, *et al*, 2003; Jaattela, Wissing, *et al*, 1998; Nanbu, Konishi, *et al*, 1998; Powers, Clarke, and Workman, 2008; Soti, Nagy, *et al*, 2005; Wei, Zhao, *et al*, 1994; Zaarur, Gabai, *et al*, 2006) strengthens this hypothesis and argues that during cellular transformation the activity of HSF1 is strongly up-regulated.

### **2.3.2. iaRNA<sup>HSF</sup> is a novel HSF inhibitor *in vitro* and *in vivo***

Here I describe the *in vivo* utility of an aptamer that targets the highly conserved HSF DNA binding domain. I engineered a potent HSF inhibitor using a dimeric molecule derived from a previously selected RNA aptamer ( $K_d \sim 30\text{nM}$ ), and demonstrate that making a single molecule containing two separate aptamers improves the affinity of the molecule approximately 3 fold, iaRNA<sup>HSF</sup>  $K_d \sim 8\text{nM}$ . Using a genetically controlled expression system, I demonstrate that I can express iaRNA<sup>HSF</sup> at high levels and inhibit HSF activity under normal and stress conditions *in vivo*. Whenever a ligand such as an aptamer is used *in vivo* to study and manipulate the function of a protein, it is important to know whether the intended target is the only molecule recognized by the ligand or drug. Here, I used a functional rather than binding assay to

demonstrate that iaRNA<sup>HSF</sup>'s binding is specific to HSF. The abnormalities that arise from the expression of iaRNA<sup>HSF</sup> in *Drosophila* are effectively suppressed by HSF co-expression and not a control protein like GFP. Since flies express a single HSF protein, this result suggests that the aptamer is exerting its effects by targeting its intended molecular surface (Zhao, Shi, *et al*, 2006). Conversely, the abnormalities observed in *Drosophila* induced by HSF over-expression are also effectively suppressed by iaRNA<sup>HSF</sup> over-expression, further supporting the specific nature of the aptamer-HSF interaction.

In mammals, HSF1 inhibition is not essential for normal cell growth, but is critical for maintenance of the transformed cancer phenotype (Dai, Whitesell, *et al*, 2007). As a second test of specificity, I show that expression of iaRNA<sup>HSF</sup> severely compromises the viability of various cancer cells without compromising the viability of non-transformed human cells. Together, the reciprocal suppression analysis of *Drosophila* phenotypes and cancer aptamer expression studies confirm the *in-vitro* specificity of the aptamer RNA to HSF (Zhao, Shi, *et al*, 2006). The fact that iaRNA<sup>HSF</sup> expression does not induce apoptosis in normal cells strongly suggests that cancer cells have distinct dependencies on the stress-sensitive chaperone network that are unique from its non-transformed counterpart.

To date, only a small class of naturally occurring molecules have been identified that effectively inhibit chaperone function and sensitize cancer cells to pharmacological treatment (Wei, Zhao, *et al*, 1994; Westerheide, Kawahara, *et al*, 2006); however, little is known about the specific mechanism of inhibition. For instance, the naturally occurring anthamycin compound geldanamycin and its derivatives (17-AAG & DMAG) have been found to inhibit Hsp90's function by blocking and inhibiting the Hsp90 ATPase domain

(Whitesell, Mimnaugh, *et al*, 1994). Geldanamycin derivatives have recently shown great promise in the treatment of a wide variety of tumors. In fact, these compounds are now in phase II clinical trials and have shown strong potential for the effective treatment of anaplastic cell lymphoma cell lines (Bonvini, Gastaldi, *et al*, 2002), acute myeloid leukemia (Minami, Kiyoi, *et al*, 2002), chronic myelogenous leukemia (Gorre, Ellwood-Yen, *et al*, 2002), prostate cancer (Vanaja, Mitchell, *et al*, 2002) and glioblastoma metastasis and cell invasiveness (Zagzag, Nomura, *et al*, 2003). Unfortunately Hsp90 inhibition by geldanamycin was also recently shown to activate osteoclast c-Src signaling and induce prostate carcinoma cells in bone tissues (Yano, Tsutsumi, *et al*, 2008). This unexpected cancer relapse may be due in part to the genetic plasticity and general ability of cancer cells to evade therapeutic treatment by the development of drug resistance (La Rosee, Corbin, *et al*, 2002). The fact that HSF1 inhibition induces apoptosis among various cancer cells without eliciting such a response in non-malignant cells (Figure 2.10) (Dai, Whitesell, *et al*, 2007) emphasizes the importance of identifying and isolating novel HSF1 inhibitors to increase the array of therapeutics available that are aimed at combinatorial cancer therapy.

### **2.3.3. HSF regulates the expression and activity of Hsp83 (Hsp90) chaperonin complex**

In this thesis, I have provided previous evidence and further demonstrated that HSF1 activity is critical for the proper expression of *Hsp83* during *Drosophila* animal development. This HSF1 requirement was first suggested by the fact that cytological site 63B which corresponds to the Hsp83 locus shows the highest HSF1 occupancy of any site in the *Drosophila*

genome, as well as the fact that *Hsp83* is the most highly-expressed protein under normal growth conditions, accounting for 1-2% of the total protein content *in vivo* (Lindquist, 1980; Brugge, Erikson, and Erikson, 1981; Oppermann, Levinson, *et al*, 1981). As shown here, either iaRNA<sup>HSF</sup> or HSF1 RNAi expression in *Drosophila* causes reduced levels of constitutive *Hsp83* expression. *Hsp83* has a general role in biological processes such as spermatogenesis, protein trafficking, signal transduction, cytoskeleton organization, and cell survival pathways (Castrillon, Gonczy, *et al*, 1993; Chakraborty, Koldobskiy, *et al*, 2008; Chen, and Balch, 2006; Lange, Bachi, *et al*, 2000; van der Straten, Rommel, *et al*, 1997; Yue, Karr, *et al*, 1999); therefore, it is likely that by decreasing *Hsp83* mRNA levels via iaRNA<sup>HSF</sup> expression results in abnormal traits that are observed when *Hsp83* activity is reduced by specific point mutations (Rutherford, and Lindquist, 1998), albeit at much higher frequencies than previously observed among *Hsp83* hemizygotes. Collectively, my data suggest that in *Drosophila* *Hsp83* is an HSF1 primary target of activation during normal conditions as well as highly responsive to HSF1 inhibition during animal development.

In human cancer cells, I show that HSF1 inhibition by iaRNA<sup>HSF</sup> results in a moderate reduction in the levels of Hsp90 protein (the *Drosophila* *Hsp83* homolog). It is likely that along with the duplication of Hsp90 genes in mammals, novel gene regulatory mechanisms have evolved to such a degree that Hsp90 partially requires HSF1 function for maintaining its normal levels. Nevertheless, because cancer cells require Hsp90 multi-subunit chaperone complexes that contain Hsp70 (Powers, Clarke, and Workman, 2008), and the fact that aptamer expression results in the downregulation of various Hsp70 family members, along with reduced levels of Hsp90 client proteins which

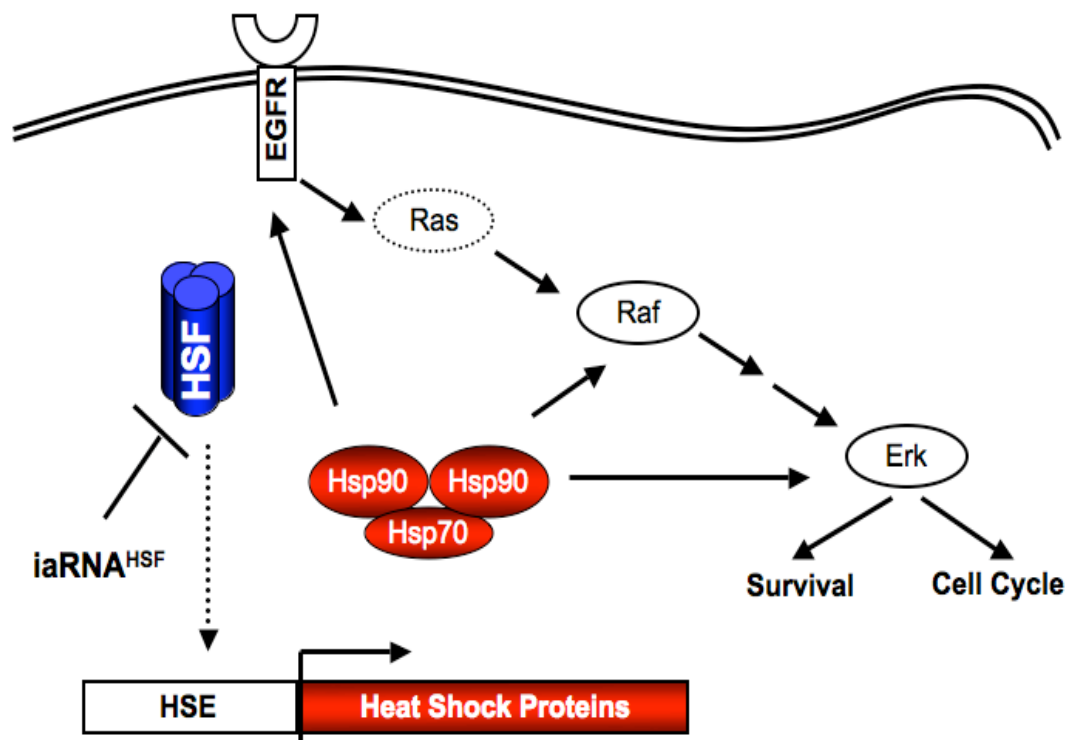
promote cancer cell survival (Erk1/2) (Figures 2.13 & 2.14), it may be concluded that HSF1 inhibition by iaRNA<sup>HSF</sup> compromises Hsp90 activity *in vivo*. The significance of this inhibition lies in the fact that Hsp90 has been found to bind and regulate the activity of hundreds of cell signaling proteins that are often aberrant in cancer (Pratt, and Toft, 2003). Additionally, anthamycin Hsp90 inhibitors affect many of the classical hallmarks of cancers that promote cancer cell survival, thus making Hsp90 an attractive therapeutic target (Hanahan, and Weinberg, 2000). Collectively, my data allows me to propose a model whereby HSF1 inhibition attenuates cell survival signaling pathways by compromising the activity of molecular chaperones in transformed cells (Figure 2.16).

#### **2.3.4. HSF regulates a buffering system that promotes adaptation to stress**

The stress response is one of the oldest and most well-conserved mechanisms that organisms have evolved to allow cells to survive constantly-changing environments. At the core of this stress response is the master regulator HSF1, which in many different organisms has been shown to regulate the expression of hundreds of genes in response to various forms of stress (Birch-Machin, Gao, *et al*, 2005; Murray, Whitfield, *et al*, 2004; Trinklein, Murray, *et al*, 2004). Cells have incorporated molecular chaperones as critical internal components of the stress response regardless of whether the stress originates from internal sources such as oxidation and protein damage that often occurs during the process of aging (Soti, and Csermely, 2003), or from external sources like nutritional balance, exposure to heavy metals, or extreme temperatures (Parsell, and Lindquist, 1993). In particular, molecular



**Figure 2.16: Proposed model for iaRNA<sup>HSF</sup> mode of action.** HSF1 inhibition by iaRNA<sup>HSF</sup> compromises cell survival (MAPK) by compromising the activity of the Hsp90/Hsp70 multi-chaperone complex. Heat shock factor (blue), molecular chaperones (red), oncogenic signaling proteins that are known to cause cellular transformation (white). Signaling proteins that are attenuated by iaRNA<sup>HSF</sup> (solid white). \*Arrows indicate direct biochemical or genetic interaction, negative signal indicates direct inhibition.



chaperones have evolved the ability to properly fold or degrade damaged proteins caused by a broad spectrum of stresses. Given the genetic instability, high adaptive capacity and elevated chaperone levels observed in nearly every type of cancer cell, it is not surprising that there exist distinct differences in the general stress response mechanism between normal and malignant cells (Murray, Whitfield, *et al*, 2004). Here, I demonstrate that HSF1 inhibition by iaRNA<sup>HSF</sup> expression results in the attenuation of highly conserved signaling pathways that often promote different classes of tumors in humans (Davis, Navolanic, *et al*, 2003; Jones, Zhang, *et al*, 2008; McCubrey, Steelman, *et al*, 2006; Navolanic, Steelman, and McCubrey, 2003; Parsons, Jones, *et al*, 2008), possibly by disrupting the functions of members of a larger chaperone network that promote cancer cell survival, including Hsp70 and Hsp90 family members (Figure 2.14) (Palotai, Szalay, and Csermely, 2008).

Overall, this study builds upon a previous *Hsp83*-directed “buffering” model (Rutherford, and Lindquist, 1998; Sollars, Lu, *et al*, 2003), and our data support the hypothesis that the master regulator HSF1 regulates the activities of a large chaperone network. In particular, HSF1 inhibition results in an altered chaperone-driven buffering system that promotes animal trait variation. In mammalian cells, HSF1 regulates a stress-sensitive buffering system that allows cells to survive and cope with stress. Such stress-sensitive buffering is central to cancer cell survival since under limiting chaperone conditions many oncogenes quickly become degraded. Given the large diversity of altered genetic backgrounds observed among different types of cancers, it will be interesting to determine how maintenance of the HSF1-regulated chaperone network, or components thereof, contribute to the maintenance of malignant phenotypes among various types of tumors. In this work, I provide an *in vivo*

approach that combines an interspecies system aimed at understanding HSF1's function during animal development and its putative role for early drug target validation. Because the HSF1 DNA linker domain is highly conserved among eukaryotes, it is likely that iaRNA<sup>HSF</sup>, or derivatives thereof, will prove to be useful in further unraveling HSF1's role in other model organisms or in diseases such as cancer.

**CHAPTER THREE: IDENTIFICATION OF THE HSF1-REGULATED**  
**CHAPERONE NETWORK IN *DROSOPHILA MELANOGASTER* AND**  
**HUMAN CELLS**

### **3.1. Introduction:**

#### **3.1.1. Identification of members of the chaperone network regulated by HSF1**

In this section, I propose various experiments that attempt to identify HSF1 regulated genes in both whole *Drosophila melanogaster* and various human cancer cells in a genome-wide manner. To identify the members of the HSF1 regulated chaperone network, I describe how the inhibition of specific HSF1-regulated genes will help unravel HSF1's general role in the maintenance of cell survival in *Drosophila* and human cancer cells. Moreover, I will describe experiments aimed toward the development of a therapeutic model system to test the efficacy of the HSF aptamer in mice. In all cases, the *Drosophila* system will be used to cross-reference the results isolated from the human data. Lastly, I will describe an ongoing collaboration with Dr. Kazunori Hirayoshi's laboratory using an RNA aptamer that targets the BTB-POZ domain of GAGA associated factor and work that I have done to determine its function *in vivo*.

#### **3.1.2. Elucidating the gene expression network for regulatory and anti-cancer phenotypes generated upon HSF1 inhibition**

The fact that iaRNA<sup>HSF</sup> binds tightly and specifically to HSF1 in both *Drosophila* and mammals allows us to exploit the complementary advantages provided by each system. Using *Drosophila* as a model system, I plan to study the general patterns of gene regulation on HSF1 targets that are well conserved between both species. A significant advantage of using *Drosophila* over other systems is the fact that flies have a single HSF1 gene and any results generated in flies should be easier to interpret as opposed to the

multiple HSF isoforms in other systems. Furthermore, performing these experiments in *Drosophila* provides a broad battery of assays not currently available in human culture models such as *in vivo* imaging techniques, and a wide collection of mutants that can be used in whole animal studies (Schwartz, Werner, and Lis, 2004; Yao, Munson, *et al*, 2006; Brand, and Perrimon, 1993). Importantly, identifying the chaperone network regulated by HSF1 provides a great starting framework for determining novel drug targets that are likely to be highly relevant in human cancer therapy.

I have demonstrated that the expression of iaRNA<sup>HSF</sup>, which inhibits DNA binding of HSF1, causes striking phenotypes in whole *Drosophila* and human cancer cells under normal growth conditions, and has potent anti-cancerous properties in various cancer cell lines (Chapter 2). In *Drosophila*, this aptamer evokes a high penetrance of developmental phenotypes that are associated with reduced expression of Hsp83/90, and its expression suppresses abnormalities induced by gain-of-function mutations in the EGFR<sup>Eip</sup> and Raf<sup>BT98</sup> oncogenes. In human cancer cell lines, HSF1 inhibition by iaRNA<sup>HSF</sup> expression severely compromises transformation-induced growth and promotes apoptosis specifically among cancer cells. These phenotypes can be attributed to the function of the key heat shock proteins Hsp70 and Hsp83/90 and their role in regulating the apoptotic and cell survival signaling pathways (Chapter 1). However, to further understand the full gamut of changes in gene expression caused by inhibiting HSF1, I propose to inhibit HSF1 with iaRNA<sup>HSF</sup> and measure the immediate changes in gene expression using the *Drosophila* and mammalian cancer cell culture model systems as discussed below.

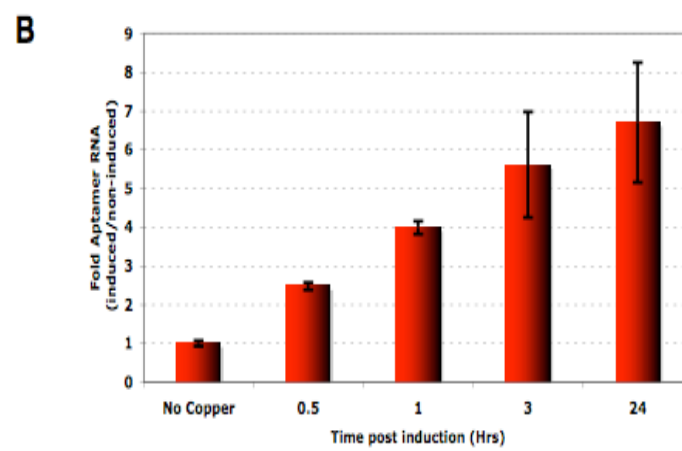
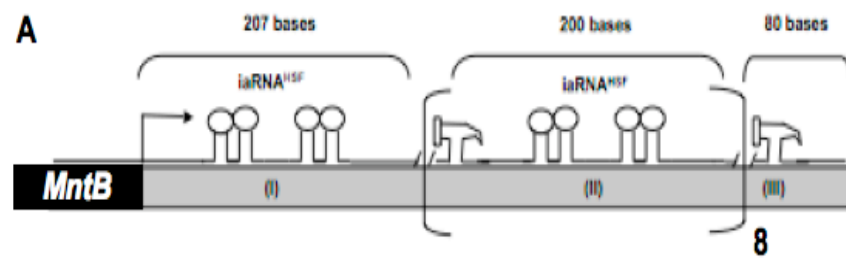
### **3.2. Surveying the effects of the HSF aptamer on global gene regulation in *Drosophila***

To test the effects of iaRNA<sup>HSF</sup> expression in *Drosophila* S2 cells, I have cloned the aptamer gene under the copper-inducible promoter, since this level of aptamer over-expression will allow us to obtain global patterns of HSF inhibition at specific time points post aptamer induction. Transient transfection experiments indicate that the addition of copper to S2 cells that contain aptamer genes results in high-level iaRNA<sup>HSF</sup> expression within the first few hours following treatment (Figure 3.1). Stable cell lines carrying these copper-inducible aptamer genes show that *Drosophila* S2 cells can effectively show a copper response in a dosage-dependent manner (Figure 3.2A) and thus induce aptamer RNAs to high cellular concentrations (Figure 3.2B). At these high levels of aptamer expression, iaRNA<sup>HSF</sup> inhibits HSF1 trans-activation of the *Hsp70* and *Hsp83* loci under both non-inducing and heat shock conditions relative to the Rev mock RNA-expressing control (Figure 3.2C). Moreover, iaRNA<sup>HSF</sup> expression inhibits HSF1 DNA binding activity as observed at the *Hsp70* loci under non-inducing conditions relative to matched control cells (Figure 3.2D). In these experiments, the relative levels of HSF1 on the *Hsp70* promoter are decreased upon iaRNA<sup>HSF</sup> expression whenever cells are treated with copper sulfate. Collectively, my results indicate that iaRNA<sup>HSF</sup> expression demonstrates potent HSF1 inhibitory activity in *Drosophila* S2 cells.

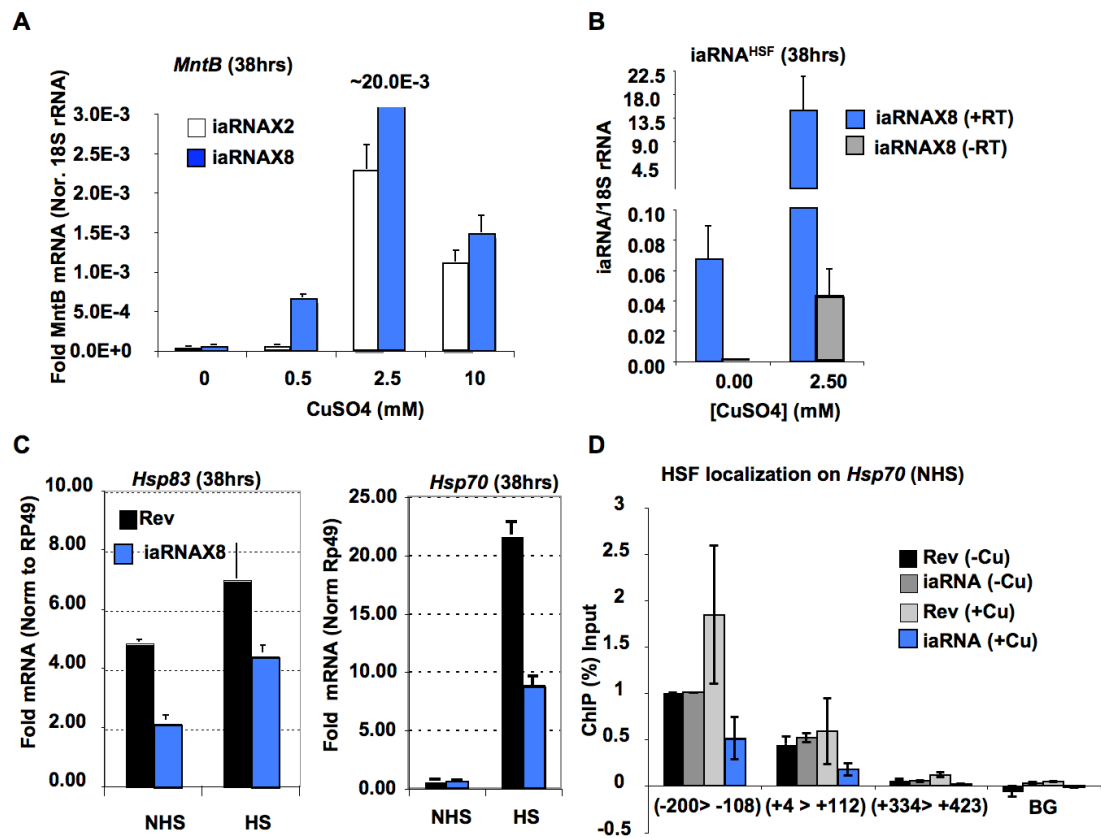
To identify genes that are regulated by HSF1, I plan to compare the various patterns of gene expression following the induction of iaRNA<sup>HSF</sup> versus a control RNA (Rev) and against cells that have been depleted of HSF by RNAi. I plan to examine this using our newly-developed GRO-seq assay



**Figure 3.1: Cloning and transient iaRNA<sup>HSF</sup> expression in *Drosophila* S2 cells.** A). Schematic representation of aptamer polymeric templates cloned downstream the copper inducible (methalothienin *MtnB*) promoter into the pDEST48 Gateway destination vector (*Invitrogen*). Here, each aptamer gene (205bp) is cloned in a head to tail manner and flanked by a self cleaving hammer-head ribozyme (hammer) to ensure release of the functional unit following a transcription cycle. B). Determination of iaRNA<sup>HSF</sup> levels in transiently transfected *Drosophila* S2 cells mock treated or treated with 5mM CuSO<sub>4</sub> at specific time points shows that the aptamer genes are responsive to copper sulfate addition (iaRNA levels were quantified using qPCR, normalized to Rp49, n=3).



**Figure 3.2: iaRNA<sup>HSF</sup> expression inhibits HSF1 activity in *Drosophila* S2 cells** (A). Addition of various concentrations of CuSO<sub>4</sub> induces the endogenous copper responsive gene (*MntB*) to various levels in two different iaRNA<sup>HSF</sup> stable *Drosophila* S2 cell lines (white=aptamer polymer of 2; and blue bars= aptamer polymer of 8 repeating units) (% total RNA values normalized to 18S rRNA, n=3). B). Addition of 2.5mM CuSO<sub>4</sub> for 38 hrs induces high level of HSF aptamer *in vivo* (blue) in stable cell lines harboring iaRNA<sup>HSF</sup> polymeric templates downstream the copper inducible promoter (*MtnA*) (total RNA is normalized to 18S rRNA; contaminating DNA is plotted in gray; n=3). C). iaRNA<sup>HSF</sup> expression inhibits HSF1 trans-activation activity under non-heat shock (NHS) and heat shock (HS) conditions *in vivo*. Black bars correspond to the mRNA levels in cell lines harboring a control reverse aptamer sequence (Rev), while the blue bars correspond to iaRNA<sup>HSF</sup> expressing cell lines (total mRNA values are normalized to Rp49, n=3). D). Induction of iaRNA<sup>HSF</sup> expression in stable *Drosophila* S2 cells (blue bars) inhibits HSF1 DNA binding activity to *Hsp70* loci under NHS conditions compared to control cells (light gray= uninduced iaRNA<sup>HSF</sup>; black= uninduced Rev; gray= induced Rev sequence. % ChIP signal corresponds to total antibody signal normalized to the no Ab control, n=2).



(Core, Waterfall, and Lis, 2008), which examines nuclear run-on products by massive parallel sequencing to provide a highly-sensitive mapping of the position, amount, and orientation of transcriptionally-engaged RNA polymerases across entire genomes. Because this method examines nascent transcription, it is particularly effective in detecting first-order changes in gene expression and will allow tracking of the immediate changes in gene regulation that accompany expression of the HSF aptamer. This is superior to standard genome-wide expression assays since GRO-Seq it is not affected by the steady state 'background' RNA levels commonly detected during template hybridization in microarray platforms.

The genes showing altered levels of expression using both the HSF aptamer and RNAi methods relative to control RNA expressing cells will be the primary candidates for further investigation. I do not expect the results of the two methods to be necessarily congruent since the RNAi knockdown of HSF requires several days of incubation and therefore may produce secondary effects. The RNA aptamer method of inhibition should provide a more direct means to examine the primary effects of HSF inhibition given that the aptamer can be induced within a few minutes. However, the RNAi results that match those obtained with the RNA aptamer will further validate those candidates of greatest interest.

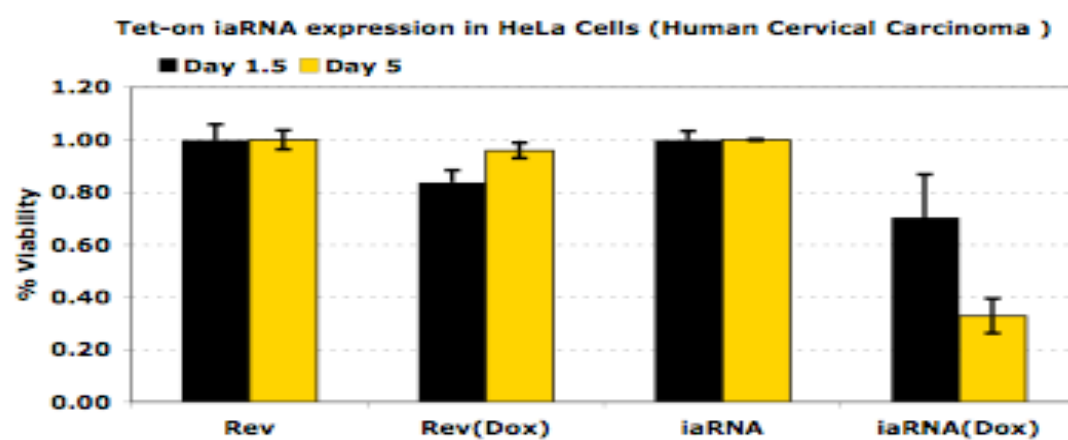
After identifying HSF1 regulated genes in *Drosophila*, I plan to classify these genes by molecular function, if known, using the GO-terms provided on the UCSC genome browser, and determine if these genes have any mammalian homologs with similar functions in an attempt to understand their putative function in transformation.

### **3.3. Surveying the effects of the HSF aptamer on global gene regulation during carcinogenesis in mammalian cells**

As a first step towards understanding the involvement of HSF1 in tumorigenesis, I propose to identify the primary targets that are regulated by HSF1 in primary NHEK (normal human epidermal keratinocytes), IMR90 (primary human lung fibroblast), and HeLa cells, since these lines are from epithelial descent (Lazo, 1988), and the results obtained from these cell lines might reveal new insights regarding the role of HSF1 during carcinogenic transformation.

To study the effects of regulated iaRNA<sup>HSF</sup> expression, I have cloned and generated HeLa cells that contain iaRNA<sup>HSF</sup> genes downstream the inducible tetracycline promoter (Figure 3.3). Treatment of HeLa cells with doxycycline as a substitute for tetracycline results in the effective induction of apoptosis within a few days following doxycycline treatment relative to control cells (Rev) or parental cell lines that are not treated with doxycycline. Unfortunately, the stable aptamer cell lines that I generated in these experiments cells began to show reduced doubling rates when compared to parental or control cell lines, a defect that is presumably due to the leaky expression of the tet-on system. In spite of this difficulty, I discovered that this expression system does not induce any toxic effects on its own and can be easily adapted for the regulated expression of aptamer genes. Therefore, in order control the aptamer genes more stringently, I have begun to clone the aptamer genes downstream the tet-repressor promoter. I have already generated multiple clonal HeLa cell lines that express the tet-repressor protein

**Figure 3.3: Induction of iaRNA<sup>HSF</sup> from a tetracycline promoter induces apoptosis in HeLa cells.** Comparison of HeLa cell viability among stable cells that harbor both a control (Rev) aptamer sequence or the HSF aptamer (iaRNA<sup>HSF</sup>) gene under the tetracycline inducible promoter as a function of time. Here, stable cells were established by transfecting either the control or the HSF aptamer into the pDest30 Tet<sup>on</sup> inducible vector, and selecting for G418 resistant cells (*Invitrogen*). After the selection procedure, induction of the reporter genes was accomplished by the addition of 1 µg/ml doxycycline to stable TREX-Hela Tet<sup>on</sup> cells. General cellular viability in these experiments was determined at 36 hrs (black) or 120 hrs using trypan blue staining.

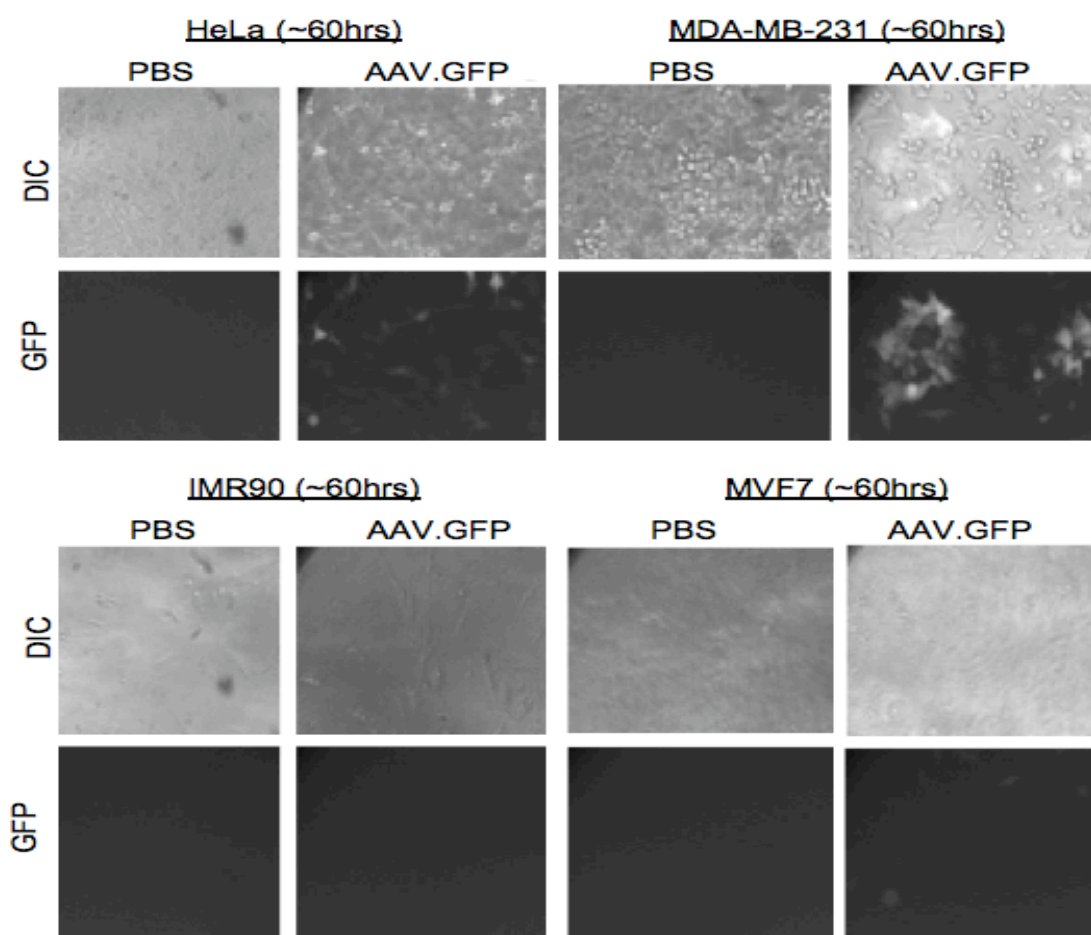




to be used as acceptor cell lines in tet-off experiments that have been named pTRE tight tet-off clones 1-24. Using the pTRE Tight tet-off cells, I plan to introduce and generate cervical carcinoma cells that contain iaRNA<sup>HSF</sup> genes under tetracycline control and determine the changes in gene expression that occur during the early phases of HSF1 inhibition relative to control RNA expression. This experiment can be expanded to compare the changes in gene expression in NHEK, IMR90, and multiple human cancer lines by taking advantage of the high transduction efficiency provided by a retroviral delivery system (pRev.TRE *Clontech*) or adenovirus (AAV) particles containing aptamer expressing genes. Preliminary experiments show that AAV gives high transduction efficiency among a variety of human cancer cells (Figure 3.4). By comparing the iaRNA<sup>HSF</sup> effect on HSF1 inhibition in two normal and one transformed backgrounds (NHEK & IMR90 vs HeLa), we will be able to obtain a snapshot of the changes in gene expression which are dependent on HSF1 activity in epithelial cells using the GRO-seq method. In a similar manner to the *Drosophila* S2 cell studies above, we can complement the above experiments and deplete HSF1 using shRNAi as an independent source of HSF1 inhibition. At the same time, we can confirm the expression results generated from the GRO-seq assays and choose candidate genes in order to analyze the relative levels of HSF1 occupancy, and their expression levels among aptamer expressing or control RNA expressing cells.

This strategy will likely identify genes whose expression is affected by the aptamer and whose function may promote tumor formation, thus identifying specific candidates and possible pathways that play a role in the development of a tumor phenotype. Thus, this genome-wide approach has great potential to reveal the cascade of transcriptional regulatory events that depend on HSF1

**Figure 3.4: Comparison of transduction efficiency of a high titer AAV.GFP in various human culture cells.** The following cell lines, MCF7-human breast cancer cell line, MDA-MB-231 human breast cancer cell line, HeLa-human cervical carcinoma, and IMR90-human primary lung fibroblast were transduced with an AAV virus that expresses a GFP reporter gene. In comparison to the total cellular population (DIC), the MDA-MB-231 and HeLa cells show the highest transduction efficiency compared to the MCF7 and IMR90 cells (see GFP channel). \*Infection was allowed to proceed for ~3hrs, then the virions were washed away and cells were grown for approximately 60 hours before visualizing cells under the DIC and GFP channels.



*\*Cells infected for 3hrs.*

transcriptional activity. A potential limitation of this approach is that there may be lots of changes and only a subset is critical for the phenotype. Given the high degree of functional conservation between human and flies, we can cross-reference the global profiles of each organism as a method of narrowing the subset of genes that help promote the cancer phenotype.

Beyond the scope of these proposed experiments, it is tempting to consider that each of these conserved HSF1 regulated genes may play an important role in the maintenance of tumors. Therefore, each HSF1-regulated gene identified from the proposed genome-wide analyses could become a putative drug target, against which we can begin selecting novel aptamers. Each of those aptamer candidates could be introduced into various cancer cell lines as described in Chapter 3 in an attempt to identify those aptamers that produce slow growth, apoptosis, or terminal differentiation among cancer cell lines relative to controls to identify genuine modulators of carcinogenesis. The goal is to isolate aptamers with potent and highly specific anti-tumor properties and categorize their activity in the context of a broad spectrum of human cancers with the long term goal of obtaining a global transcription profile of various types of cancers and an assessment of their overall dependency on the chaperone network.

## **CHAPTER FOUR: PERSPECTIVES**

#### **4.1. Examining effects of aptamers on mammalian tumor formation**

To establish an aptamer based therapeutic model, future experiments should focus on understanding whether local instillation of cancer cells that contain small-molecule-inducible forms of the aptamer in mice as mentioned in chapter 3. In these experiments, cancer cells containing the small-molecule-inducible form of the aptamer should be used in xenografts experiments in mice and the transformation capacity, and metastatic potential of the cancer cells should be closely evaluated in the absence or presence of aptamer expression. In these experiments, aptamer expression will be controlled by introducing small-activating molecules (i.e. tetracycline or doxycycline) within the food, or by direct injection of the small-activating molecules into the bloodstream of the mice.

As an alternative approach toward establishing an aptamer based therapeutic model, aptamer expression can be delivered into mature tumors using a 'gene therapy approach', where recombinant viruses that contain aptamer-expressing genes are injected directly into cancerous tissues/sites in mice. In this approach, the efficacy of the aptamer will be tested by comparing the size of the treated tumors at specific time points relative to a paired RNA control. We can expect that those tumours that are sensitive to HSF1 inhibition will respond well to the aptamer based viral therapy. However, for those types of cancers that are resistant to HSF1 inhibition by the aptamer, it will be interesting to determine if these particular types of cancers are more susceptible to traditional forms of cancer therapy such as radiation or chemotherapeutic treatment.

Overall, this analysis should reveal the potential therapeutic value of the HSF1 aptamer during the various stages of tumor progression in specific

human cancer cells, at the same time, allow us to predict the therapeutic value of the aptamer against a wide collection of pre-existing tumors caused by specific mutagens (or deregulated proteins) in a mammalian system *in vivo*.

#### **4.2 Dissecting the mechanism of aptamer action in *Drosophila***

The powerful *Drosophila* genetic system allows for a thorough investigation of the mode of action of aptamer expression in a non-transformed genetic background. In this system, we can explore the changes in gene expression induced by HSF1 inhibition by iaRNA<sup>HSF</sup> in the context where specific mutations such as inactivated tumor suppressors or hyperactivated oncogenes can be easily and quickly manipulated in a whole animal. By expressing aptamers in such specified genetic backgrounds, we will be able to analyze the relationship between HSF1 and specific HSF1-regulated gene targets that are known to promote oncogenesis in mammals. Given that in cancerous cells contain hundreds of genetic mutations and aberrations, one advantage of our *Drosophila* model of tumorigenesis is that we will be able to analyze the effects of specific cancer inducing mutations in an a 'clean' genetic background, thereby allowing us to study the genetic interactions between HSF1 and specific cancer causing mutations. At the same time, we will be able to score the hierarchy of each of these genetic interaction and determine how each contribute to deregulated cell growth using our tissue specific tumor models in flies, the eyes and wings as mentioned in chapter 2.

### **4.3 Morphological effects of aptamers on specific oncogenic backgrounds**

I have previously demonstrated that aptamers and specific cancer mutants can be expressed in specific tissues or developmental stages in *Drosophila* by placing each target under the tight genetic control of the UAS-Gal4 system (Chapter 2). In brief, this strategy works for our existing HSF aptamer by examining its effects on gain-of-function mutants within the MAPK signaling pathway, Raf and EGFR gain-of-function mutants. As shown in Figure 2.9, I have demonstrated that the morphological abnormalities induced by the oncogenic mutant protein are effectively suppressed by HSF aptamer expression, demonstrating that this strategy provides a convenient and quantitative measurement of aptamer function *in vivo*. These assays can be used with additional aptamers that have putative anti-oncogenic potential. Not only can single oncogene mutations be used in such assays, but given the ease and power of *Drosophila* genetics, one can also introduce multiple specific combinations of such mutations to elucidate the relationship of signaling nodes and their dependence on the chaperone network.

### **4.4 Global regulatory changes caused by aptamers on specific oncogenic backgrounds**

In *Drosophila*, we can explore the global changes in gene expression between aptamer-expressing cells either lacking or expressing specific oncogenes in order to assess the relationship between a particular oncogene and the chaperone network using the GRO-seq protocol. The large amount of expression information from each two-by-two matrix comparing normal and cancer-modeled cells with and without aptamer will yield genes that will be



divided into classes. In the first case, we will identify the genes most affected by inhibiting the entire network of proteins whose expression is dependent on HSF1 activity by expressing iaRNA<sup>HSF</sup>. With the availability of our newly-generated aptamers to individual HSF1-regulated proteins, many of which we expect will be chaperone network members (Chapter 2), we can identify how each member of the network contributes to the maintenance of the entire network by classifying the subsets of genes affected upon inhibition. The effects of specific oncogenic backgrounds on these patterns of gene expression in cells that express aptamers to HSF1 (or specific chaperones) will identify those genes that are either affected or indifferent to this "chaperone therapy". This information will sharpen current strategies for the treatment of various classes of tumors since this analysis will identify new classes of gene that contribute to the deregulated cell growth associated with specific cancers. For example, we envision that an aptamer to HSF1 will affect a large number of genes, while an aptamer to a specific chaperone will affect only a small subset. If both have comparable abilities to reverse the effects of a specific oncogene, then clearly the aptamer targeting a specific chaperone is the preferred reagent as a therapeutic lead. At the same time, but beyond the scope of this analysis, those genes that differ between oncogenic and normal cells but are not changed upon aptamer expression can be classified as putative novel drug targets worthy of aptamer selection specific to that particular abnormal genetic background or cancerous condition. The number of two-by-two matrices generated would depend on the number of aptamers with anti-oncogenic activity identified and the number of oncogenic backgrounds available in the *Drosophila* community. For starters, we will also focus on a few of the best-characterized oncogene and tumor suppressor

mutants in *Drosophila*, including members of the receptor tyrosine kinase family such as EGFR, members of the Raf mitogen-activated signaling pathway, and classic tumor suppressors such as p53 and Rb. Lastly, a similar analysis will be performed in non-transformed human cells by introducing specific cancer-causing mutations to compare expression profiles with and without the introduction of specific aptamer RNAs.

In conclusion, the proposed battery of assays will allow for the assessment of the modes of action of a particular aptamer in a single mutant background or in backgrounds of specific combinations of mutants. The interplay of both the mammalian and *Drosophila* systems provides an efficient and cost-effective means of assessing the anti-cancer properties and mode of action of RNA aptamers, and their utility as therapeutic inhibitors *in vivo*.

## **CHAPTER FIVE: MATERIALS AND METHODS**

\*This chapter includes the techniques and reagents used in my experiments.

### **Oligonucleotides and other reagents**

A single iaRNA<sup>HSF</sup> unit was constructed in two parts by extending 50pmoles of each of the following primer sets (I & II; III & IV) in 100ul using a single round PCR reaction:

I).5'CCGCTCGAGTGACGTTGGCATCGCGATACAAAATTAAGTTGAACGCG  
AGTTCTTCGGAAT,

II).5'GGCCGGAATTCAAGGAGTATGACGAAGGCAGTTGAATTCCGAAGAAC  
TCGCGTTCAACTT

III).5'GGCCGGAATTCAACTGCCTTCGGGCATCGCGATACAAAATTAAGTTG  
AACGCGAGTTCTTGGAGGCTCGACGTCT,

IV).5'CGCGTCGACGTTTCGTCCTCACGGACTCATCAGTAGCGAAACCACA  
TCGCTAGACGTCGAGCCTCCAAGAACTCG.

After extension, the resulting products were visualized by EtBr staining and extracted from an 8% native polyacrylamide matrix. Then, each template was digested with EcoRI (Invitrogen), and ligated together resulting in a product that contains two individual aptamer genes (Apt-HSF1) and a self-cleaving hammerhead ribozyme (iaRNA<sup>HSF</sup>). The iaRNA<sup>HSF</sup> product was cloned into pstBlue-blunt cloning vector pstBlue.iaRNA<sup>HSFX1</sup> (Novagen).

### **Construction of synthetic genes**

Repetitive head-to-tail iaRNA<sup>HSF</sup> genes were created by sub-cloning iaRNA<sup>HSFX1</sup> into a Gateway donor vector (pDONR221.iaRNA<sup>HSFX1</sup>) by amplifying the iaRNA<sup>HSFX1</sup> sequence from pstBlue.iaRNA<sup>HSFX1</sup> using primers

containing the AttB1F and AttB2R Gateway cloning sequences (Invitrogen): 5' AAGTTTGTACAAAAAGCAGGCTTCGGATCCAGAATTCGTGATC and 5' GGGGACCACTTTGTACAAGAAAGCTGGGTTAGCCTAGGTCGACG.

Because each iaRNA<sup>HSF</sup> unit is flanked by the complementary asymmetric XhoI and Sall restriction sites at the 5' and 3' ends, respectively, we can use the general Gateway cloning strategy as a way to select for correctly ligated tandem iaRNA<sup>HSF</sup> repeats. In this method, a single iaRNA<sup>HSFX1</sup> unit is first amplified from pDONR221.iaRNA<sup>HSFX1</sup> via PCR and the resulting amplicon is cut with either Sall or XhoI before the cut products are combined and ligated together. Using this scheme, only those products that are in proper head-to-tail orientation contain the required Gateway AttB sites in the 5' and 3' ends (AttB1F.iaRNA<sup>HSFX2</sup>.AttB2R) needed for creation of a Gateway compatible *Drosophila* transformation expression vector, pUAS.iaRNA<sup>HSFX2</sup>. Using the polymer of 2 as template and repeating the dimerization strategy creates a polymer of 4, p{UAS.iaRNA<sup>HSFX4</sup>, w+}. Overall, geometric progression of polymeric length is achieved in each subsequent round of dimerization.

### ***Drosophila* strains**

Parental iaRNA<sup>HSF</sup> animals were created by injecting *Drosophila*<sup>w1118</sup> embryos with p{UAS.iaRNA<sup>HSFX8</sup>, w+} and p{UAS.iaRNA<sup>HSFX16</sup>, w+} transformation vectors and screening the progeny of F<sub>1</sub> females for animals that contain the mini-white gene when crossed to a doubled balanced CSX fly line: CyO(2); TM6(3); Xasta(2,3). Sites of p-element insertions were determined genetically by continuous backcrossing to the CSX stock, resulting in homozygous fly lines that contain aptamer genes in various chromosomes:

1. UAS.iaRNA<sup>HSFX8</sup>(I)
2. UAS.iaRNA<sup>HSFX16</sup>(I)
3. UAS.iaRNA<sup>HSFX8,16</sup>(I)
- 4.

UAS.iaRNA<sup>HSFX8</sup>(II) 5. UAS.iaRNA<sup>HSFX16</sup>(II) 6. UAS.iaRNA<sup>HSFX8,16</sup>(II) 7. UAS.iaRNA<sup>HSFX16</sup>(III). To express iaRNA<sup>HSF</sup>, we crossed homozygous UAS.iaRNA<sup>HSF</sup> stock animals with various Gal4 sources purchased from the *Drosophila* Stock Center (Bloomington, IN): 6983 (Salivary Gland Gal4), 5138 (Ubiquitous tubulin Gal4). Systemic iaRNA<sup>HSF</sup> expressing strains were created by isolating F<sub>1</sub> females from aptamer parental containing aptamer genes in the second chromosome (UAS.iaRNA<sup>HSF8</sup> & UAS.iaRNA<sup>HSF8,16</sup>) mated to 5138 animals. Heterozygous F<sub>1</sub> males were then mated to CSX females, and the resulting F<sub>2</sub> animals that contained both aptamer genes and Gal4 protein (UAS.iaRNA<sup>HSF</sup>/CyO; Tub.Gal4/Sb) were isolated and isogenized to create true breeding aptamer expressing lines. Other Bloomington stocks used in this study include 5693 (Hsp83<sup>e6D</sup> antimorphic mutant) and 5743 (Duplication 61F7-F8; 64B10-12). The pUAS.eGFP strain was given as a gift from Dr. Antonio Garcia-Bellido's laboratory, while strains expressing the gain of functions EGFr<sup>Eip</sup> and Raf<sup>BT98</sup> mutants were also gifts kindly provided by Dr. Marc Therrien.

### ***In vitro* binding assays**

Internally labeled <sup>32</sup>P-UTP iaRNA<sup>HSF</sup> was transcribed from a iaRNA<sup>HSFX1</sup> PCR template containing a 5'-T7 promoter using a Maxi-script Kit (Ambion). Electrophoretic motility shift assays (EMSA) were performed by addition of increasing molar amounts of purified GST-HSF protein to limiting amounts of <sup>32</sup>P-iaRNA<sup>HSF</sup> (<1nM) using the following binding conditions: 25mM Tris HCl, 75mM KOAc, 0.5Mm MgCl<sub>2</sub>, 10% glycerol, pH=7.4, and allowing complexes to form for 0.5 hrs at 25°C. RNA/protein complexes were separated in 6% native agarose gel (3mM Tris HCl, 200mM glycine, 0.5mM MgCl<sub>2</sub>). Competition

experiments were performed by incubating increasing molar amounts of cold iaRNA<sup>HSF</sup> together with labeled Hsp83 or Hsp70 promoter DNAs followed by the addition of 50nM purified HSF-GST for 30 minutes at room temperature. The resulting complexes were then separated on 2% agarose gels (0.5X TAE, 0.5mM MgCl<sub>2</sub>) or passed over nitrocellulose filters used for quantification in filter-binding assays. Promoter DNA sequences were amplified from *Drosophila* genomic DNA using the following primer sequences: *Hsp83*-449F: 5'-ACTTGACTGGGCTTGTAGCAGGTT, *Hsp83*+114R:5'-TTCTGGATGCCAGGGATGCAACTT, *Hsp70*-200F:5'-TGCCAGAAAGAAAACCTCGAGAAA, *Hsp70*+64R:5'-CTGCGCTTGTTTGTTTGCTTAGCT

### **RNA quantification**

Total RNA was extracted from whole animals using Trizol reagent (Invitrogen). Quantification of the relative transcript levels was determined by oligodT<sub>20</sub> reverse transcription, followed by real time PCR analysis (RT-qPCR) using the following primer sets:

Rp49+141F: 5'-CCCAAGGGTATCGACAACAGA,  
 Rp49+204R: 5'-CGATGTTGGGCATCAGATACTG,  
 18S +417F: 5'-TGACGAAAAATAACAATACAGGACTCA,  
 18S +569R: 5'-CAGACTTGCCCTCCAATTGG,  
 iaRNA<sup>HSF</sup> F: 5'-TGGTTTCGCTACTGATGAGTCCGT,  
 iaRNA<sup>HSF</sup> R: 5'-GCAGTTGAATTCCGAAGAACTCGC,  
*Hsp70Ab*+2155F:5'- GGTGCGACTAAGGCCAAAGAGTCTA,  
*Hsp70Ab* +2266R:5'- TCGATCGAAACATTCTTATCAGTCTCA,

*Hsp83 + 3628F*: 5'-GCGACCAGTCGAAACAAACAACCA,

*Hsp83 + 3732F*: 5'-AACTCGGCCGTAGTAAACTCAG,

*Hsp26+580F*: 5'-CAAGGTTCCCGATGGCTACA,

*Hsp26+667R*:5'-CTGCGGCTTGGGAATACTGA

### **Statistical analysis**

All statistical analyses were calculated using student's t-test.

### **Immunofluorescent assays of polytene chromosomes**

Salivary glands were dissected from third stage instar larvae in 0.5X Grace's medium. Chromosomes were spread, fixed onto slides and immunostained using antibodies targeting HSF, GAGA Factor (GAF) as described previously in Schwartz *et al* (Schwartz, Werner, and Lis, 2004).

### **Morphological studies**

Aptamer-expressing animals were scored for phenotypic abnormalities using a dissecting microscope. Pictures were taken using an 8.0Mb Nikon digital camera. Quantification of morphological abnormalities was calculated by quantifying abnormal size or area using the ImageJ software (NIH).

### **Tissue Culture**

iaRNA<sup>HSFX8</sup> was subcloned into Gateway pDEST48 (Invitrogen) and stable *Drosophila* S2 cells were stably selected by maintaining cells in 6 ug/ml Blasticidin reagent. iaRNA<sup>HSF</sup> was induced using 0.5mM CuSO<sub>4</sub>.

Mammalian IMR90, and HeLa cells were grown in E-MEM low glucose medium (ATCC) supplemented with 10% FBS, 1X Pen/Strep in 5% CO<sub>2</sub>.



293T cells were grown in DMEM high glucose medium (ATCC) supplemented with 10% FBS, 1X Pen/Strep in 5% CO<sub>2</sub>. MDA-MB-231 cells were grown in RPMI medium (ATCC) supplemented with 10% FBS, 1X Pen/Strep in 5% CO<sub>2</sub>. BE-(2)-M17 cells were grown in 1:1 EMEM low glucose medium (ATCC) + F12 medium (ATCC) supplemented with 10% FBS, 1X Pen/Strep in 5% CO<sub>2</sub>. Upon confluency, cells were trypsinized and passed into fresh medium according to ATCC instructions.

For all constitutive level expression experiments, iaRNA<sup>HSFX8</sup> was subcloned into Gateway pDEST51 (Invitrogen) and semi-stable cells were maintaining cells in 0.5-6ug/ml Blasticidin reagent depending on the cell type. For generation of Tet-on stable cells, iaRNA<sup>HSFX8</sup> was subcloned into Gateway pDEST30 (Invitrogen) and stable cells were generated by transfecting iaRNA<sup>HSF</sup> into HeLa-Trex (Invitrogen) and maintaining the cells in 100ng/ml G418 until drug resistant cells appeared. iaRNA<sup>HSF</sup> was delivered into mammalian cells using Lipofectamine (Invitrogen) or Lipofectine (Quiagen) transfection reagents according to the manufacturer's instructions.

### **Apoptotic Assays:**

Apoptosis was observed by quantifying the number of HeLa cells containing fragmented nuclei as visualized by DAPI staining under the microscope. In these experiments, parental, iaRNA<sup>HSF</sup> or Rev RNA control cells were transfected and apoptosis was observed for after 96 hrs of aptamer expression. In these experiments, non-transfected iaRNA<sup>HSF</sup> or RNA control cells were subsequently discarded from the population by culturing the cells in 6 µg/ml blasticidin and washing the cells 24 hrs after transfection. Only the blasticidin-resistant cells were used throughout subsequent assays.

**Transformation Assays:**

Transformation was determined by the ability of cells to grow on soft agar. In these experiments,  $6 \times 10^3$  semi-stable cells were plated on 6-well dishes in appropriate medium supplemented with 3% agarose (Type VII, Sigma A4018) over a warm layer of pre-solidified medium containing 6% agarose (Type VII, Sigma A4018). On day 7 an additional layer of medium containing 3% agarose was added to each of the wells. Colonies were analyzed under the light microscope on day 14.

**Western Blots:**

Western blots were performed according to conventional protocols. PVDF membranes were blocked using 5% BSA and membranes were incubated primary antibodies overnight at 4°C.

*Primary Antibodies:* G6PDH was purchased from Sigma (A9521). PARP DBD was a kind gift from Lee Kraus' group. All other antibodies were purchased from Cell Signaling Inc.: EGFR~p (No. 2234), Erk1/2~p (No. 9146), total Erk1/2 (No. 9102), Hsp40 (No. 4868), Hsp60 (No. 4870), Hsp70 (No. 4872), Hsp90 (No. 4875), HSF1 (No. 4356), Calnexin (No. 2433), PDI (No. 2446), Bip (No. 3177).

Secondary antibodies were used according to proper immunoreactivity.

**RNAi:**

Templates for generating dsRNA were generated by amplifying genomic DNA using the following primer sets containing a T7 promoter and dsRNA was generated by *in vitro* transcription. RNA was DNase I treated,

phenol:chloroform extracted, and the RNA was heated and cooled, producing dsRNA.

T7Hsp83+378F:

5'-TAATACGACTCACTATAGGGTTCCATGATCGGTCAGTTCGGTGT,

T7Hsp83-1048R:

5'-TAATACGACTCACTATAGGGCGTACAGCTTGATGTTGTTGCGCT

T7HSF F:

5'GAATTAATACGACTCACTATAGGGAGAGCCTTCCAGGAGAATGCA,

T7HSFR:

5'GAATTAATACGACTCACTATAGGGAGAGCTCGTGGATAACCGGTC

### **RNAi Treatment**

~1x10<sup>6</sup> *Drosophila* S2 cells were incubated with 10 µg dsRNA targeting HSF and Hsp83 for 5 days using genes containing T7 promoter which targeted each sequence amplified from *Drosophila* genomic DNA.

## APPENDIX

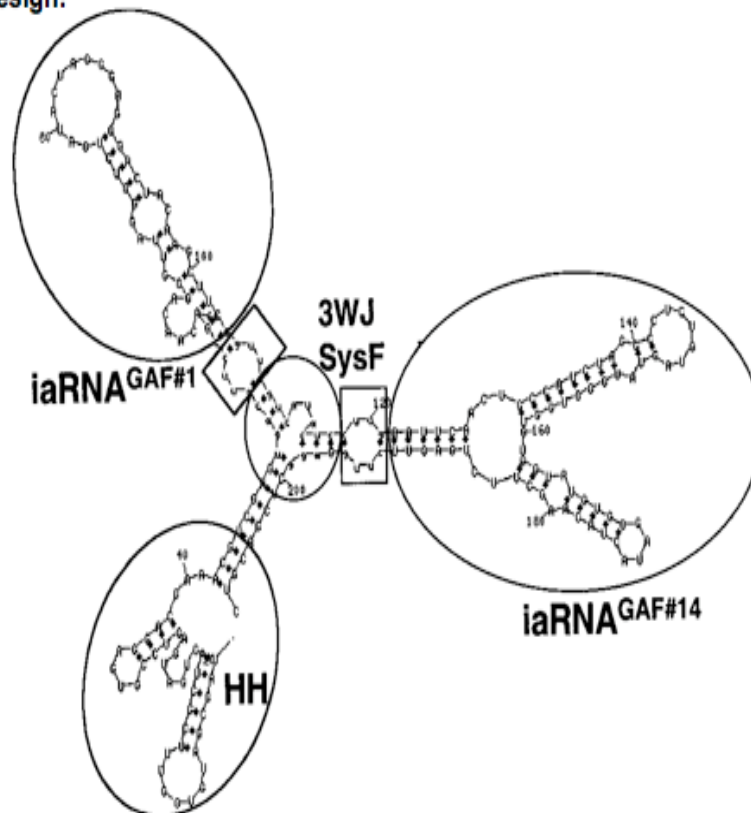
### **Dissecting the role of GAGA Associated Factor using RNA Aptamers targeting the BTB-POZ domain**

As part of an ongoing collaboration with Dr. Kazunori Hirayoshi's group (Kyoto University, Japan), I engineered a divalent RNA aptamer (iaRNA<sup>GAF1-14</sup>) comprised of two previously-selected aptamers that target the GAP BTB-POZ domain (Figure A.1). Biochemical assays from Dr. Hirayoshi's group showed that each of these aptamers bind avidly to GAF with an approximate dissociation constant of 30nM, and can inhibit *Hsp70* expression *in vitro* transcription assays. Using their system, Dr. Hirayoshi's group showed that iaRNA<sup>GAF1-14</sup> has an approximate K<sub>d</sub> of 5nM (Figure A.2A). In a similar manner as the HSF1 aptamer described in Chapter 2, I made tandem head-to-tail polymeric constructs of iaRNA<sup>GAF1-14</sup> of 8 repeating units, each flanked by a self-cleaving hammerhead ribozyme, and cloned this long template downstream of either the inducible *MntB* promoter or the Gal4 (UAS) inducible promoter for generating stable *Drosophila* S2 lines or expression of the aptamer in whole *Drosophila*, respectively.

Expression of the aptamer via addition of 2.5mM copper sulfate to cells effectively reduces induction of *Hsp70* following a heat stress relative to control expressing cells (Figure A.2B). In whole *Drosophila*, I induced aptamer expression in flies (F<sub>1</sub>) by mating animals that constitutively and ubiquitously express Gal4 protein with homozygous aptamer (non-expressing) animals (F<sub>0</sub>). As heterozygotes, iaRNA<sup>HAF1-14</sup> expressing animals do not display any gross abnormalities, yet by increasing the aptamer dosage by generating true-breeding fly lines, I see various abnormal phenotypes that phenocopy known

**Figure A.1: Design of an RNA aptamer targeting GAGA factor (GAF),**  
**iaRNA<sup>GAF1-14</sup>.** (A). M-fold prediction of iaRNA<sup>GAF1-14</sup> secondary structure and  
direct sequence. (Red Letters indicate the aptamer sequence; Yellow Letters  
indicate the hammer head ribzyme).

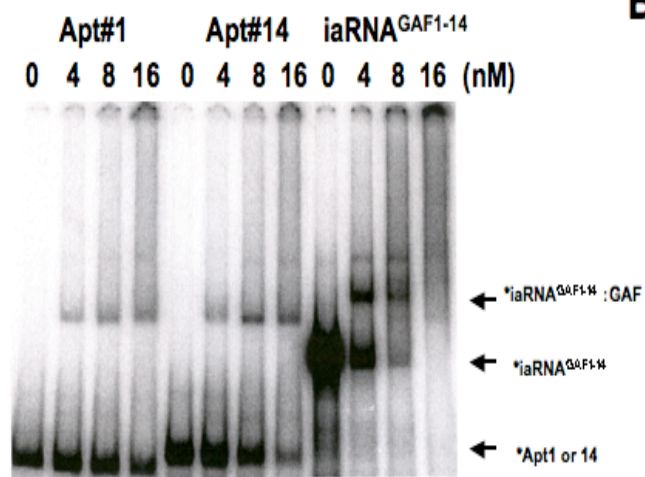
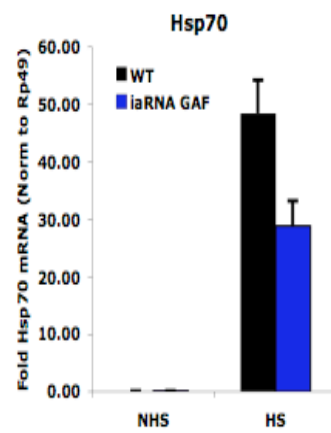
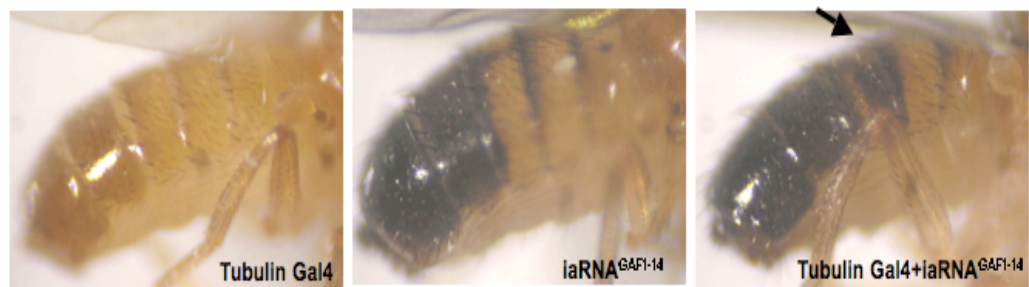
iaRNA<sup>GAF1-14</sup> Design:



5' cTCGAGt gacgttcc **GA CAACAGGGTT AGGGTCTGA TACTAGGGAG GGACTACAAG CTTC**  
 ggttcg tcatact cct tgAATTCAAC TG CCATCTAg gcctctgtag tatgggtggt gtggtatgtg ggatACTACA  
**AGCTTCT** GAG TTcttgGagg CTCGAC **GTCT AGCGATGTGG TTTCGCTACT GATGAGTCCG**  
**TGAGGACGAA AC GTCGAC**-3'

**Figure A.2: iaRNA<sup>GAF1-14</sup> binds to GAF *in vitro* and *in vivo*.**

A). Gel shift analysis shows that radiolabelled divalent GAF construct (iaRNA<sup>GAF1-14</sup>) binds more avidly to increasing concentrations of purified GAF protein than the single aptamer isoforms, Apt1 & Apt14, and displays an apparent dissociation constant of  $K_d \sim 5\text{nM}$ . B). Expression of iaRNA<sup>GAF1-14</sup> from stable *Drosophila* S2 cells inhibits the heat shock induction of *Hsp70* mRNA relative to control RNA expressing cells (WT). \*In these experiments, cells harboring the aptamer genes under the copper inducible promoter were treated with 2.5mM copper sulfate for 36 hours before treating them with 37C for 20 min and isolating RNA (fold mRNA is normalized to the internal control Rp49 gene, n=3). C). High level constitutive expression of iaRNA<sup>GAF1-14</sup> (right) in whole flies induces an abnormal abdomen segment phenotype (arrow) in whole animals relative to parental non-iaRNA<sup>GAF1-14</sup> expressing animals (middle), or compared to Gal4 expressing animals (left). Here, the iaRNA<sup>GAF1-14</sup> expressing animals (right) are homozygous within the second chromosome and contain a polymer of 8 aptamer genes and also express Gal4 protein under the regulation of the tubulin promoter on the third chromosome. (Left: Tubulin Gal4 parent; Middle: WT iaRNA<sup>GAF1-14</sup> parent; Right: Tubulin Gal4 + iaRNA<sup>GAF1-14</sup>).

**A****B****C**



GAF point mutants (Bejarano, and Busturia, 2004). In this regard, , iaRNA<sup>HAF1-14</sup> expression results in *Drosophila* animals with abnormal pupal shapes, pupal lethality, or transformed abdominal segments (Figure A.2C), although many of these phenotype occur at low frequency (<5%).

Besides the abdominal segment phenotype observed in iaRNA<sup>GAF1-14</sup> expressing flies, I find that true breeding iaRNA<sup>GAF1-14</sup> animals display abnormal longitudinal veins where the primary vein lacks many of the marginal hairs. Moreover, I find that such true breeding stocks are hard to maintain because these vials have low fecundity. In an attempt to address the specificity of the GAF aptamer and suppress these aptamer induced phenotypes, I tried over-expressing GAF-GFP protein, however, I found that GAF-GFP over-expression results in animal lethality by itself, and this phenotype is not suppressed by the aptamer (data not shown). These results suggest that the aptamer is not present at sufficient levels to effectively inhibit the aberrant functions of GAF-GFP over-expression, or alternatively, that iaRNA<sup>GAF1-14</sup> has non-specific properties and binds to other proteins *in vivo*. In this regard, I compared the relative homology of the GAF BTB-POZ domain against all other annotated protein domains within the *Drosophila* genome, and found that many *Drosophila* proteins contain a BTB-POZ domain, including the *longitudinal lacking* protein (p val= $5 \times 10^{-22}$ ) and *fruitless* (p-val= $2 \times 10^{-15}$ ) gene. It is likely that iaRNA<sup>GAF1-14</sup> binds to and inhibits these proteins resulting in not only the abnormal wing margin defect, the low fecundity rates, and also the abnormal mating behaviours observed among iaRNA<sup>GAF1-14</sup> expressing male flies. In this last scenario, iaRNA<sup>GAF1-14</sup> males display a similar defect that is also present among *fruitless* mutants where the males tend to court and attempt to mate with other male flies.

Having demonstrated that iaRNA<sup>GAF1-14</sup> inhibits GAF trans-activation function *in vitro* (Kazunori *et al.*, in preparation), I measured the levels of *Hsp26*, *Hsp70* and *Hsp83* genes under both non-inducing and heat shock conditions in constitutively aptamer expressing animals but found no significant change in the levels of heat shock gene expression among iaRNA<sup>GAF1-14</sup> expressing or control animals in either condition (data not shown). However, recent findings suggest that stable-copper inducible iaRNA<sup>GAF1-14</sup> *Drosophila* S2 cell lines have decreased *Hsp70* mRNA transcripts relative to control RNA expressing cells. It is likely that these differences are due to higher levels of aptamer RNA in culture cells when expressed from the copper inducible promoter as compared to the constitutive level expression in *Drosophila* animals mentioned previously. Future experiments should focus on the role of GAF in maintaining the promoter in an open conformation, its role in promoting HSF1 binding to heat shock elements at *Hsp70*, regulating chromatin architecture, and the establishment of a transcriptionally-engaged yet paused polymerase using the copper inducible iaRNA<sup>GAF1-14</sup> *Drosophila* S2 cells.

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